O-GlcNAc Peptide Epoxyketones Are Recognized by Mammalian Proteasomes

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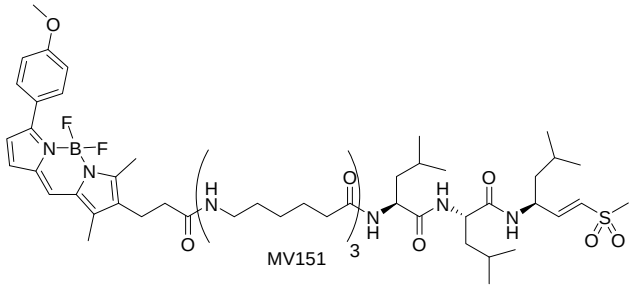
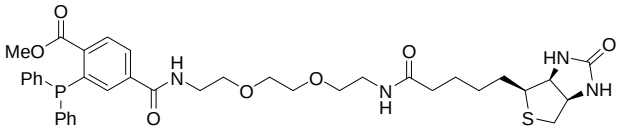
Structure of MV151 and biotin modified phosphine:





Biotin modified phosphine

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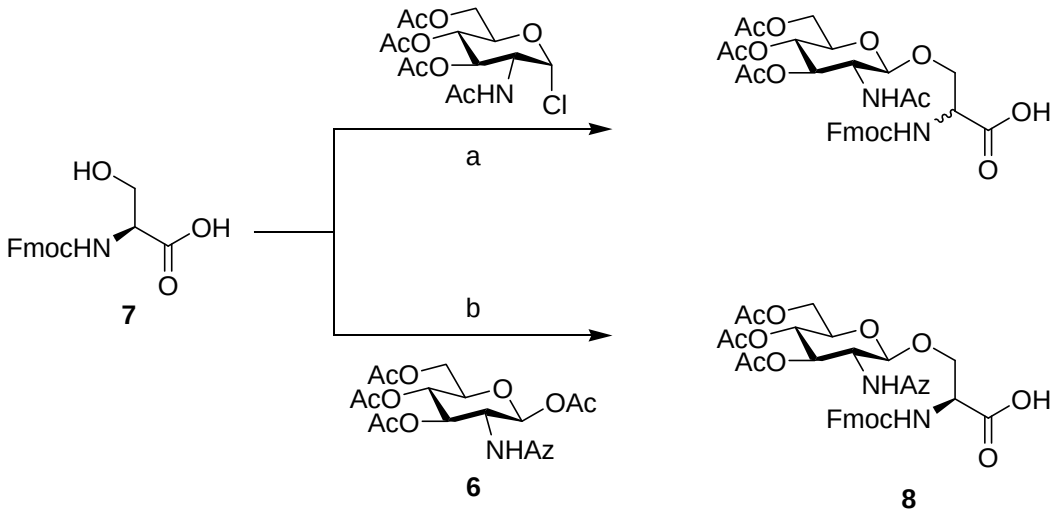
Synthesis

We reasoned that both the peracetylated (**1** and **3**) and the unprotected (**2** and **4**) derivatives could be prepared using the block coupling strategy. The N-terminal glycopeptide parts of the inhibitors could be synthesized employing solid phase peptide synthesis, and these peptides could then be condensed to the warhead. Subsequent deprotection should give glycosylated proteasome probes **1-4**. For the on-resin synthesis of glycopeptides **11** and **12**, suitably protected glycosylated serine analogues were

needed. Known Fmoc-Ser(OGlcNAc)-OH was synthesized in one step from Fmoc-Ser-OH and 2-acetylacetamido-3,4,6-tetra-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride as described in literature (Scheme 1).[1] The NMR-data of the obtained Fmoc-Ser(OGlcNAc)-OH was in accordance with the literature. LC-MS data however showed two peaks in a ratio of 1 to 1.6, possibly caused by epimerization of the c-α of serine. Fmoc-Ser(OGlcNAc)-OH was used as such in the synthesis for probes **3** and **4**. Fmoc-Ser(OGlcNAz)-OH **8** was prepared as depicted in Scheme 1. Lewis acid mediated condensation of known 2-azidoacetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-glucopyranose **6**[2] with commercially available Fmoc-Ser-OH **7** gave glycosylated building block **8**. Although purification of **8** proved to be difficult, starting compounds **6** and **7** are easily accessible and their condensation afforded **8** in reasonable yield and as a single diastereomer, according to LC-MS and NMR.

Scheme 1. Synthesis of glycosylated Fmoc-building blocks

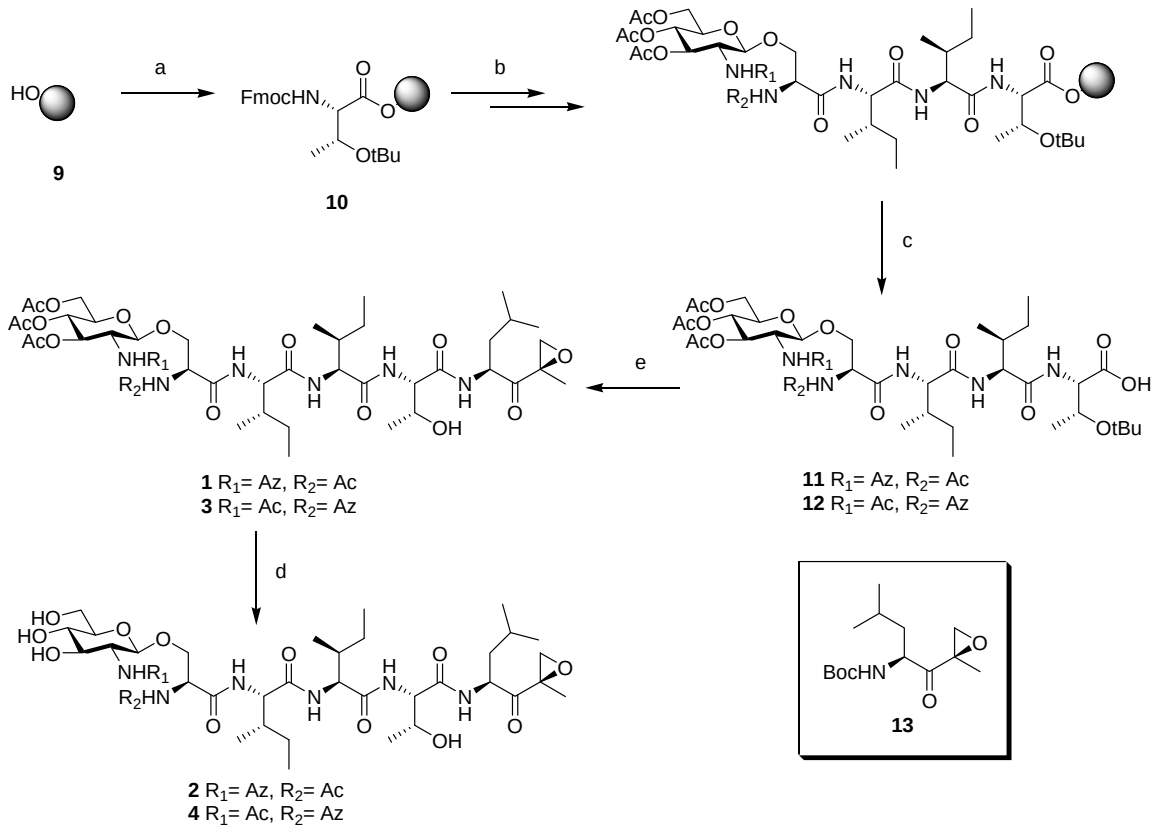


Reagents and conditions: (a) $HgBr_2$, CH_2ClCH_2Cl, reflux; (b) $BF_3{\cdot}Et_2O$, MeCN, 48h, 54%.

Synthesis of proteasome probes 1-4

Proteasome probes **1-4** were synthesized as depicted in scheme 2. MBHA resin equipped with a HMPB linker **9** was loaded with FmocThr(OtBu)OH **10**. Solid phase peptide synthesis followed by mild acidic cleavage from the resin afforded glycopeptides **11** and **12** which were directly used in a condensation with known warhead **13**[3]. To this end, the *tert*-butoxycarbonyl protective group of **13** was removed with trifluoroacetic acid. Peptides **11** and **12** were activated using HCTU in the presence of diisopropylethylamine (DiPEA), after which they were added to deprotected **13**. Aqueous work-up followed by removal of the *tert*-butyl protective group of the threonine using 50% TFA in CH_2Cl_2 gave crude proteasome probes **1** and **3**. HPLC-purification allowed separation of the epimeric mixture, which was formed by the use of diastereomerically unpure Fmoc-Ser(OGlcNAc)-OH and/or epimerization during block coupling. Probes **1** and **3** were deactylated by treatment with a catalytic amount of NaOMe in MeOH affording glycosylated probes **2** and **4**.

Scheme 2. Synthesis of glycosylated proteasome probes **1-4**.

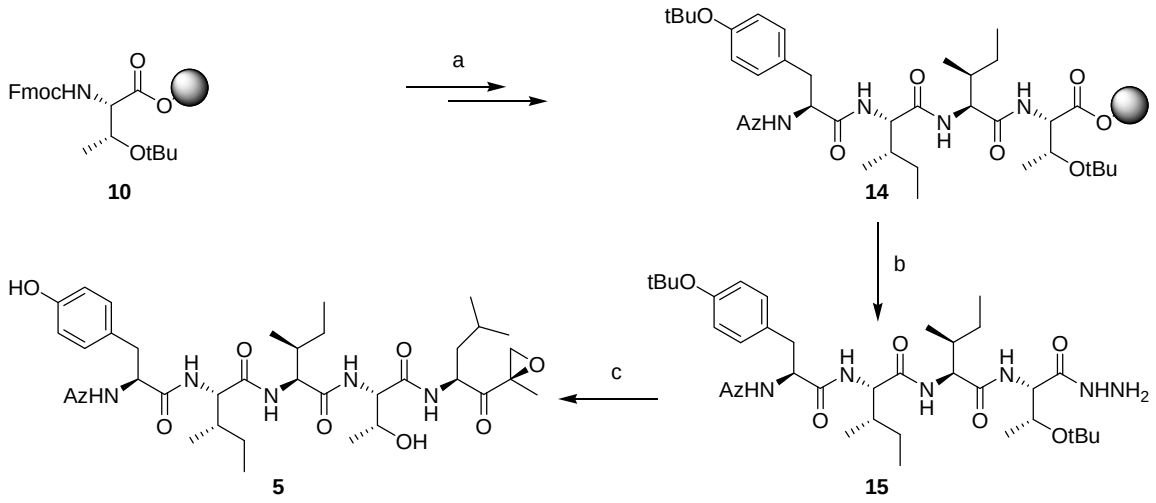


Reagents and conditions: (a) Fmoc-Thr(OtBu)-OH, DIC, DMAP; (b) i) 20% piperidine in NMP; ii) Fmoc-Ile-OH, Fmoc-Ser(OGlcNAc)-OH or **7**, HCTU, DiPEA, NMP; (c) 1% TFA/CH_2Cl_2, **11**: 84%, **12**: 71%; (d) i) **13**, HCTU, DiPEA; ii) 50% TFA/CH_2Cl_2, **1**: 30%, **2**: 26%; (e) NaOMe/MeOH, **3**: 50%, **4**: 41%.

The P5 position of probes **1-4** is sterically encumbered. To study the influence of the steric bulk on binding of the probe, probe **5** was synthesized (Scheme 3). In this probe, the carbohydrate core is replaced by a tyrosine residue. To this end, peptide **14** was synthesized on resin. Hydrazide **15** was obtained form peptide **14** by esterification with trimethysilyl diazomethane followed by treatment with hydrazine. The resulting

hydrazide was converted to the corresponding acyl azide after which it was condensed with warhead **13**. Removal of the protective groups gave probe **5** in good yield.

Scheme 3. Synthesis of epoxomicin equipped with a bulky residue at the P5 position



Reagents and conditions: (a) i) 20% piperidine in NMP; ii) Fmoc-Ile-OH, Fmoc-Tyr(OtBu), HCTU, DiPEA, NMP; (c) 1% TFA/CH_2Cl_2, quant; (d) i) **13**, HCTU, DiPEA; ii) 50% TFA/CH_2Cl_2, 63%.

General Procedures:

All reagents were commercial grade and were used as received unless stated otherwise. Biotine modified phosphine and NAG-thiazoline were synthesized as described.[4,5] Building blocks **6** and **13** were synthesized using a modified literature procedure.[1,3] Diethyl ether (Et_2O), ethyl acetate (EtOAc), light petroleum ether (PE) and toluene (Tol) were purchased from Riedel-de Haën. Acetonitrile (MeCN), dichloroethane, dichloromethane (CH_2Cl_2), dimethylformamide (DMF), methanol (MeOH) and pyridine (pyr) were obtained from Biosolve. Before use CH_2Cl_2 was refluxed over CaH_2 for 2h

and distilled. Molecular sieves 3Å were flame dried prior to use. All reactions were performed under an inert atmosphere of Argon unless stated otherwise. Solvents used for flash chromatography were of pro analysi quality. Flash chromatography was performed on Screening Devices silica gel 60 (0.04 – 0.063 mm). TLC-analysis was conducted on DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254 nm) were applicable and by spraying with 20% sulfuric acid in ethanol followed by charring at ~150°C or by spraying with a solution of $(NH_4)_6Mo_7O_{24}\cdot H_2O$ (25 g/l) and $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ (10g/l) in 10% sulfuric acid in water followed by charring at ~150°C. ^1H and ^{13}C NMR spectra were recorded on a 400/100 MHz spectrometer. Chemical shifts (δ) are given in ppm relative to the residual solvent peak or tetramethylsilane as internal standard. Coupling constants are given in Hz. All given ^{13}C spectra are proton decoupled. High resolution mass spectra were recorded by direct injection (2 μL of a 2 μM solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass spectrometer equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60000 at *m/z* 400 (mass range *m/z* = 150-2000) and dioctylpthalate (*m/z* = 391.28428) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). LC-MS analysis was performed on a HPLC system with a standard C_{18} (4.6 mmD × 250 mmL, 5μ particle size) column (detection at 200-600 nm) in combination with buffers A: H_2O, B: MeCN and C: 0.5% aq. TFA and coupled to a mass spectrometer with ESI. For RP-HPLC purifications an automated HPLC system equipped with a C_{18} (5μm 250×10 mm) column was used. The applied buffers were A: 0.1% TFA in H_2O, B: MeCN.

1,3,4,6-tetra-*O*-acetyl-2-azidoacetamide-2-deoxy-β-D-glucopyranose (6)

1,3,4,6-Tetra-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranose[6] (19 g, 50 mmol) was dissolved in CH_2Cl_2, cooled to 0°C. Chloroacetic anhydride (12 g, 70 mmol) and Et_3N (16.7 mL, 120 mmol) were added. After 1h, TLC analysis showed complete conversion. The solution was washed with $NaHCO_3$ (sat. aq.), 1M HCl, dried (Na_2SO_4), concentrated and redissolved in DMF. Sodium azide (13.0 g, 200 mmol) was added and the solution was stirred overnight. After removal of the volatiles, the crude product was dissolved in CH_2Cl_2, washed with $NaHCO_3$ (sat. aq.), 1M HCl and dried (Na_2SO_4). The crude product was purified by column chromatography ($CH_2Cl_2 \rightarrow$ 2% MeOH/CH_2Cl_2) giving known **6** (79%, 17.07 g, 39 mmol). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 6.57 (d, *J* = 9.1 Hz, 1H), 5.80 (d, *J* = 8.7 Hz, 1H), 5.29 (t, *J* = 9.9, 9.9 Hz, 1H), 5.14 (t, *J* = 9.6, 9.6 Hz, 1H), 4.32-4.20 (m, 2H), 4.14 (dd, *J* = 12.5, 2.1 Hz, 1H), 3.91 (s, 2H), 3.87 (ddd, *J* = 9.8, 4.5, 2.1 Hz, 1H), 2.12 (s, 3H), 2.10 (s, 3H), 2.05 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 170.9, 170.5, 169.29, 169.26, 167.0, 92.1, 72.8, 72.1, 67.7, 61.6, 53.1, 52.5, 20.8, 20.6, 20.5.

Fmoc-Ser(OGlcNAz)-OH (8)

Fmoc-Ser-OH **7** (247 mg, 0.75 mmol) and azidoacetylglucosamine **6** (325 mg, 0.75 mmol) were coevaporated with toluene (3×) before being dissolved in MeCN/CH_2Cl_2 (11 mL, 2.5/3 v/v). Activated 3Å molecular sieves were added and the mixture was stirred for 30 min before it was cooled to 0°C. $BF_3{\cdot}Et_2O$ (171 μL) was added after which the reaction was stirred for 72h at room temperature. The reaction was quenched with Et_3N,

extracted with 1M HCl and brine, dried over Na_2SO_4 and concentrated. Silica gel column chromatography (CH_2Cl_2 (0.1% AcOH)→ 2% MeOH/CH_2Cl_2 (0.1% AcOH)) followed by LH20 size exclusion chromatography afforded title compound **8** (54%, 280 mg, 0.40 mmol). ^{1}H NMR (400 MHz, $CDCl_3$/MeOD) δ ppm 7.78 (d, *J* = 7.48 Hz, 2H), 7.67 (d, *J* = 7.13 Hz, 2H), 7.40 (t, *J* = 7.39, 7.39 Hz, 2H), 7.33 (ddt, *J* = 7.62, 7.62, 2.62, 0.98 Hz, 2H), 5.32 (t, *J* = 9.90, 9.90 Hz, 1H), 5.03 (t, *J* = 9.65, 9.65 Hz, 1H), 4.80 (d, *J* = 8.40 Hz, 1H), 4.51-4.33 (m, 3H), 4.31-4.21 (m, 2H), 4.19 (dd, *J* = 10.76, 4.66 Hz, 1H), 4.13 (dd, *J* = 12.32, 1.77 Hz, 1H), 3.93 (dd, *J* = 10.75, 3.36 Hz, 1H), 3.90-3.81 (m, 2H), 3.81-3.73 (m, 3H), 2.07 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$/MeOD) δ ppm 170.9, 170.6, 170.1, 169.3, 168.4, 156.2, 143.18, 143.15, 140.6, 127.0, 126.4, 124.3, 124.3, 119.2, 99.8, 71.5, 71.0, 68.4, 68.1, 66.4, 61.3, 53.6, 51.4, 46.4, 46.1, 19.46, 19.40. LC/MS: R_t 8.35 min; linear gradient 10→90% B in 13.5 min; ESI/MS: *m/z* = 698.20 $(M+H)^+$. FT-IR: ν_{max}(neat)/cm^{-1} 3318.9, 2110.0, 1744.5, 1695.0, 1535.8, 1451.4, 1368.5, 1222.0, 1039.2. HRMS: $(M+H^+)$ calcd for $C_{32}H_{35}N_5O_{13}$ 698.23041 found 698.23095. $[\alpha]_D^{23}$ + 15° (c = 1, $CHCl_3$).

Boc-Leucine epoxy ketone (13)

$NaHCO_3$ (11.76 g, 140 mmol) was dissolved in H_2O (20 mL) and cooled to -10°C. Trifluoroacetone (13.44 mL, 150 mmol) and (1-isobutyl-3-methyl-2-oxo-but-3-enyl)-carbamic acid tert-butyl ester[3] (5 mmol in 25 mL MeCN) were added followed by the portionwise addition of oxone (21.5 g, 35 mmol). The reaction was diluted with Et_2O, extracted, the organic layer was dried (Na_2SO_4) and concentrated in vacuo. Column chromatography (PE→ 8% EtOAc/PE) afforded warhead **13** (38%, 0.516 g, 1.9 mmol).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 4.98 (d, *J* = 8.6 Hz, 1H), 4.32 (dt, *J* = 9.7, 9.3, 3.0 Hz, 1H), 3.30 (d, *J* = 4.9 Hz, 1H), 2.89 (d, *J* = 5.0 Hz, 1H), 1.78-1.69 (m, 1H), 1.52 (s, 3H), 1.41 (s, 9H), 1.19 (ddd, *J* = 13.9, 10.4, 4.1 Hz, 2H), 0.97 (d, *J* = 6.5 Hz, 3H), 0.94 (d, *J* = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 209.4, 155.5, 79.5, 58.8, 52.1, 51.2, 40.2, 28.1, 24.9, 23.2, 21.1, 16.6.

Solid phase peptide synthesis

MBHA resin functionalized with HMPB **9** (2 g, 1.2 mmol/g) was coevaporated with dry dichloroethane. The resin was solvated in CH_2Cl_2 and Fmoc-Thr(OtBu)-OH (1.43 g, 3.6 mmol), N,N'-diispropylcarbodiimide (0.616 mL, 3.9 mmol) and DMAP (20 mg) were added. After 3h, the resin was filtered, washed and subsequently applied to another coupling cycle. The loading was determined at 0.86 mmol/g. Fmoc-Thr(OtBu)-resin **10** was solvated in NMP after which the appropriate amino acids were condensed employing the following consecutive steps: deprotection by the use of 20% piperidine in NMP (v/v), washing with NMP (3×) and CH_2Cl_2 (3×), condensation of the amino acid (4 equiv.) under the agency of HCTU (4 equiv.) and DiPEA (8 equiv.) followed by shaking for 1.5 h, washing with NMP (3×) and CH_2Cl_2 (3×). The reactions were monitored by kaisertest and LC/MS. For the condensation of Fmoc-Ser(OGlcNAz)-OH **8** and Fmoc-Ser(OGlcNAc)-OH 1.5 equivalents of amino acid were used instead of 4 equivalents and these amino acids were coupled overnight. Double couplings were performed when incomplete coupling was observed. After coupling of the glycosylated amino acid, the resin was deprotected with 20% piperidine in NMP, washed and capped. Ac_2O (5 equiv.) and DiPEA (5 equiv.) in NMP was used to cap peptide **11**. Peptides **12** and **14**, which

contains an *N*-terminal azido acetyl, were capped with bromoacetyl bromide (5 equiv.) in the presence of DiPEA (10 equiv.) in NMP for 1h. The resin was washed and sodium azide (5 equiv.) in DMF was added followed by overnight shaking. The peptides were released from the resin by treatment with 1% TFA/CH_2Cl_2. An equal amount of toluene was added after which the volatiles were removed giving the crude peptides.

Ac-Ser(OGlcNAz)-Ile-Ile-Thr(OtBu)-OH (11)

Solid phase synthesis as described above afforded peptide **11** (84%, 0.758 g, 0.841 mmol). LC/MS: R_t 7.12 min; linear gradient 10→90% B in 13.5 min; ESI/MS: *m/z* = 901.07 $(M+H)^+$. HRMS: $(M+H^+)$ calcd for $C_{39}H_{64}N_8O_{16}$ 901.45130 found 901.45264.

Ac-Ser(OGlcNAz)-Ile-Ile-Thr-LeuEK (1)

Boc-LeuEK **13** (81 mg, 0.3 mmol) was dissolved in 50% TFA/CH_2Cl_2. After 30 min, the volatiles were removed under reduced pressure. Residual traces of TFA were removed by coevaporation with toluene (3×) giving the corresponding TFA salt. Peptide **11** (224 mg, 248 μmol) was dissolved in DMF. The solution was cooled to 0°C before HCTU (113 mg, 274 μmol), DiPEA (165 μL, 0.946 mmol) and the freshly prepared TFA·LeuEK were added. After 2h LC/MS analysis showed completed conversion. The reaction mixture was concentrated, redissolved in CH_2Cl_2, washed with $NaHCO_3$ (sat. aq.), dried (Na_2SO_4) and concentrated. Subsequently, the tert-butyl protective group of the threonine was removed. Therefore, the crude peptide was dissolved in TFA/CH_2Cl_2 (4 mL, 1:1 v/v). TLC analysis showed complete conversion to a more polar product after which the reaction was concentrated coevaporated with toluene (3×) and purified by RP-HPLC (linear gradient

40%→50% B in 10 min) furnishing the major epimer of acetylated GlcNAz-proteasome probe **1** (30%, 74 mg, 74 μmol). ^{1}H NMR (400 MHz, $CDCl_3$/MeOD) δ ppm 8.23 (d, *J* = 9.2 Hz, 1H), 7.99 (d, *J* = 7.5 Hz, 1H), 7.90 (d, *J* = 7.7 Hz, 1H), 7.74-7.69 (m, 2H), 7.44 (d, *J* = 7.6 Hz, 1H), 5.25 (t, *J* = 10.0, 10.0 Hz, 1H), 5.01 (t, *J* = 9.6, 9.6 Hz, 1H), 4.68-4.50 (m, 3H), 4.37-4.23 (m, 4H), 4.14 (ddd, *J* = 12.2, 5.7, 1.9 Hz, 1H), 4.08 (td, *J* = 11.3, 5.6, 5.6 Hz, 1H), 4.00-3.89 (m, 2H), 3.88 (d, *J* = 2.6 Hz, 2H), 3.82 (dd, *J* = 11.1, 6.0 Hz, 1H), 3.77 (m, 1H), 3.26 (d, *J* = 5.1 Hz, 1H), 2.92 (d, *J* = 5.1 Hz, 1H), 2.07 (s, 3H), 2.02 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H), 1.92-1.78 (m, 2H), 1.76-1.66 (m, 1H), 1.64-1.49 (m, 3H), 1.47 (s, 3H), 1.40-1.18 (m, 6H), 1.16 (dd, *J* = 6.4, 2.6 Hz, 3H), 0.98-0.86 (m, 18H). ^{13}C NMR (100 MHz, $CDCl_3$/MeOD) δ ppm 209.3, 173.5, 173.4, 173.0, 172.2, 172.1, 171.9, 171.7, 171.0, 170.3, 101.8, 73.8, 73.1, 69.9, 69.8, 69.4, 68.3, 63.2, 60.0, 59.5, 59.5, 59.2, 54.9, 54.4, 53.06, 53.01, 51.6, 40.4, 38.3, 38.0, 37.4, 26.1, 26.1, 25.8, 23.7, 22.5, 21.5, 20.8, 20.7, 20.7, 19.8, 17.0, 15.9, 15.8, 11.4, 11.4. FT-IR: ν_{max}(neat)/cm^{-1} 3288.5, 2964.3, 2112.4, 1749.4, 1632.8, 1537.6, 1463.2, 1372.4, 1223.7, 1044.2. LC/MS: R_t 7.72 min; linear gradient 10→90% B in 13.5 min; ESI/MS: *m/z* = 998.27 $(M+H)^+$. HRMS: $(M+H^+)$ calcd for $C_{44}H_{71}N_9O_{17}$ 998.50407 found 998.50523.

Ac-Ser(OGlcNAz)-Ile-Ile-Thr-LeuEK (2)

Acetylated probe **1** (34 mg, 34 μmol) was dissolved in MeOH (1 mL). Subsequently, a catalytic amount of NaOMe was added. After 1h, TLC-analysis showed complete conversion. The solution was neutralized with Amberlite IR-120 H^+, filtered and concentrated. Purification by silica gel column chromatography (CH_2Cl_2 → 5% MeOH/ CH_2Cl_2) gave probe **2** (50%, 16.50 mg, 18.9 μmol). Reverse phase-HPLC (linear gradient

29%→39.5% B in 14 min). ^{1}H NMR (400 MHz, DMSO) δ ppm 8.06 (d, *J* = 8.7 Hz, 1H), 7.93 (d, *J* = 8.2 Hz, 2H), 7.89-7.80 (m, 2H), 7.77 (t, *J* = 8.7, 8.7 Hz, 1H), 5.04 (d, *J* = 3.4 Hz, 1H), 4.97 (d, *J* = 5.2 Hz, 1H), 4.74 (d, *J* = 4.8 Hz, 1H), 4.56 (t, *J* = 5.8, 5.8 Hz, 1H), 4.47-4.34 (m, 2H), 4.31-4.22 (m, 2H), 4.22-4.15 (m, 2H), 3.93-3.65 (m, 5H), 3.61 (dd, *J* = 10.8, 7.6 Hz, 1H), 3.50-3.39 (m, 2H), 3.18 (d, *J* = 5.1 Hz, 1H), 3.13-3.03 (m, 2H), 3.00 (d, *J* = 5.2 Hz, 1H), 1.85 (s, 3H), 1.78-1.61 (m, 3H), 1.48-1.21 (m, 7H), 1.14-1.01 (m, 3H), 0.99 (d, *J* = 6.2 Hz, 3H), 0.89 (d, *J* = 6.6 Hz, 3H), 0.85-0.75 (m, 15H). ^{13}C NMR (100 MHz, DMSO) δ ppm 207.9, 170.8, 170.6, 169.9, 169.3, 169.2, 167.2, 100.3, 76.9, 73.9, 70.4, 70.3, 67.6, 66.3, 60.9, 58.6, 57.7, 56.9, 56.8, 55.2, 52.8, 51.4, 50.6, 49.1, 36.5, 36.4, 36.3, 24.2, 24.1, 23.0, 22.3, 20.9, 19.5, 16.3, 15.1, 15.0, 10.9, 10.88, 10.84. FT-IR: ν_{max}(neat)/cm^{-1} 3273.8, 2963.4, 2107.0, 1632.8, 1542.8, 1374.9, 1069.3, 1034.0. LC/MS: R_t 6.20 min; linear gradient 10→90% B in 13.5 min; ESI/MS: *m/z* = 872.27 $(M+H)^+$. HRMS: $(M+H^+)$ calcd for $C_{38}H_{65}N_9O_{14}$ 872.47237 found 872.47345.

Ac-Ser(OGlcNAc)-Ile-Ile-Thr(OtBu)-OH (12)

Solid phase synthesis afforded peptide **12** (71%, 48 mg, 53 μmol) as an diastereomeric mixture (1 to 1.6) which was used as such. LC/MS: R_t 7.12 and 7.20 min; linear gradient 10→90% B in 13.5 min; ESI/MS: *m/z* = 901.20 $(M+H)^+$. HRMS: $(M+H^+)$ calcd for $C_{39}H_{64}N_8O_{16}$ 901.45130 found 901.45248.

Az-Ser(OGlcNAc)-Ile-Ile-Thr-LeuEK (3)

Peptide **12** (48 mg, 53 μmol) was condensed to epoxyketone **13** (18 mg, 66 μmol) and subsequently deprotected as is described for compound **1**. Reverse phase-HPLC (linear

gradient 43%→52.75% B in 13 min) afforded the major epimer of probe **3** (26%, 14 mg, 14 μmol). LC/MS: R_t 7.77 min; linear gradient 10→90% B in 13.5 min; ESI/MS: *m/z* = 998.27 $(M+H)^+$. ^{1}H NMR (400 MHz, $CDCl_3$/MeOD) δ ppm 8.36 (d, *J* = 7.9 Hz, 1H), 8.17 (t, *J* = 7.7, 7.7 Hz, 2H), 8.10 (d, *J* = 9.6 Hz, 1H), 7.93 (d, *J* = 7.8 Hz, 1H), 7.81 (d, *J* = 8.4 Hz, 1H), 5.25 (dd, *J* = 10.5, 9.3 Hz, 1H), 5.00 (dd, *J* = 10.0, 9.5 Hz, 1H), 4.90 (d, *J* = 3.7 Hz, 1H), 4.68 (t, *J* = 6.4, 6.4 Hz, 1H), 4.61-4.55 (m, 1H), 4.49-4.44 (m, 1H), 4.35-4.20 (m, 5H), 4.14-4.05 (m, 2H), 3.96 (d, *J* = 2.4 Hz, 2H), 3.95-3.91 (m, 1H), 3.80 (dd, *J* = 6.4, 3.2 Hz, 2H), 3.25 (d, *J* = 5.0 Hz, 1H), 2.93 (d, *J* = 5.1 Hz, 1H), 2.06 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 2.00 (s, 3H), 1.95-1.85 (m, 1H), 1.85-1.68 (m, 2H), 1.67-1.56 (m, 2H), 1.51 (ddd, *J* = 13.3, 9.8, 3.2 Hz, 1H), 1.46 (s, 3H), 1.40-1.15 (m, 3H), 1.13 (d, *J* = 6.3 Hz, 3H), 1.01-0.85 (m, 18H). FT-IR: ν_{max}(neat)/cm^{-1} 3289.2, 2964.8, 2111.1, 1749.7, 1638.1, 1536.4, 1369.7, 1232.1, 1040.6. HRMS: $(M+H^+)$ calcd for $C_{44}H_{71}N_9O_{17}$ 998.50407 found 998.50511.

Az-Ser(OGlcNAc)-Ile-Ile-Thr-LeuEK (4)

Probe **3** (8.0 mg, 8.0 μmol) was deacetylated as described for **2**. RP-HPLC purification (linear gradient 29%→40.3% B in 15 min) afforded probe **4** (41 %, 2.85 mg, 3.27 μmol). LC/MS: R_t 6.34 min; linear gradient 10→90% B in 13.5 min; ESI/MS: *m/z* = 872.20 $(M+H)^+$. ^{1}H NMR (400 MHz, MeOD) δ ppm 4.78 (d, *J* = 3.6 Hz, 1H), 4.64 (dd, *J* = 6.7, 5.9 Hz, 1H), 4.53 (dd, *J* = 10.6, 3.0 Hz, 1H), 4.34 (dd, *J* = 16.3, 4.8 Hz, 1H), 4.22 (d, *J* = 8.3 Hz, 1H), 4.13 (d, *J* = 8.2 Hz, 1H), 4.04 (dd, *J* = 6.2, 5.2 Hz, 1H), 3.94 (d, *J* = 2.0 Hz, 2H), 3.92-3.89 (m, 1H), 3.85-3.76 (m, 2H), 3.77-3.66 (m, 2H), 3.68-3.60 (m, 2H), 3.48 (ddd, *J* = 9.3, 5.3, 2.1 Hz, 1H), 3.36 (m, *J* = 9.8, 9.2 Hz, 1H), 3.26 (d, *J* = 5.1 Hz, 1H),

2.90 (d, *J* = 5.0 Hz, 1H), 2.02 (s, 3H), 1.84-1.74 (m, 2H), 1.74-1.48 (m, 4H), 1.47 (s, 3H), 1.38-1.28 (m, 2H), 1.26-1.11 (m, 5H), 0.98-0.83 (m, 18H). ^{13}C NMR (100 MHz, MeOD) δ ppm 209.34, 173.39, 173.36, 173.20, 171.72, 171.04, 169.43, 99.23, 73.65, 72.12, 71.93, 68.34, 68.29, 62.32, 59.89, 59.66, 59.12, 59.00, 54.65, 53.65, 52.98, 52.58, 51.54, 40.07, 37.72, 37.40, 26.00, 25.95, 25.78, 23.68, 22.95, 21.45, 19.55, 17.06, 15.67, 11.52, 11.21. FT-IR: ν_{max}(neat)/cm^{-1} 3287.7, 2965.2, 2108.7, 1636.0, 1537.9, 1376.6, 1204.8, 1133.8, 1034.1. HRMS: ($M+H^+$) calcd for $C_{38}H_{65}N_9O_{14}$ 872.47237 found 872.47340.

Ac-Tyr(OtBu)-Ile-Ile-Thr(OtBu)-OH (14)

Solid phase synthesis afforded peptide **14** (quant, 168 mg, 239 μmol). LC/MS: R_t 8.99; linear gradient 10→90% B in 13.5 min; ESI/MS: *m/z* = 704.07 $(M+H)^+$.

Ac-Tyr(OtBu)-Ile-Ile-Thr(OtBu)-NHNH$_2$ (15)

Peptide **14** (79 mg, 112 μmol) was dissolved in MeOH/Toluene (1/1, 4 mL) after which trimethylsilyl diazomethane (0.12 mL, 240 μmol, 2 M in hexanes) was added and the reaction was stirred for 1h. To quench the reaction, acetic acid was added until the yellow color disappeared. The solution was concentrated in vacuo and redissolved in degassed MeOH under argon atmosphere. Hydrazine (200 μL) was added and the reaction was refluxed for 20h. Filtration gave hydrazide **15** (67%, 54 mg, 75 μmol). LC/MS: R_t 7.54 min; linear gradient 10→90% B in 13.5 min; ESI/MS: *m/z* = 718.20 $(M+H)^+$.^{1}H NMR (400 MHz, $CDCl_3$/MeOD) δppm 6.98 (d, *J* = 8.2 Hz, 2H), 6.80 (d, *J* = 8.2 Hz, 2H), 4.88 (dd, *J* = 7.7, 4.8 Hz, 1H), 4.38-4.32 (m, 3H), 3.95-3.87 (m, 1H), 3.75 (s, 2H), 2.99 (dd, *J*

= 14.0, 4.5 Hz, 1H), 2.79 (dd, *J* = 14.7, 7.8 Hz, 1H), 1.82-1.63 (m, 2H), 1.58-1.36 (m, 2H), 1.23 (s, 9H), 1.11 (s, 9H), 1.09-1.02 (m, 3H), 0.99 (d, *J* = 6.2 Hz, 3H), 0.86-0.72 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$/MeOD) δ ppm 172.5, 172.4, 172.3, 171.8, 170.7, 168.3, 154.4, 131.8, 130.2, 124.7, 124.6, 124.5, 100.5, 79.1, 75.4, 67.4, 58.5, 58.4, 58.1, 57.4, 57.2, 54.4, 52.3, 38.1, 37.9, 37.3, 28.9, 28.4, 25.5, 25.3, 19.0, 15.6, 15.5, 11.5, 11.3.

Az-Tyr-Ile-Ile-Thr-LeuEK (5)

Hydrazide **15** (54 mg, 75 μmol) was dissolved in CH_2Cl_2/DMF (5 mL) before being cooled to -30°C. *tert*-Butyl nitrite (10 μL, 83μmol) and hydrochloric acid (53 μL, 210 μmol, 4M in dioxane) were added. TLC-analysis showed complete conversion to the acyl azide after 2h stirring at -30°C.

Leucine epoxyketone **13** (24.4 mg, 90 μmol) was dissolved in 50% TFA/CH_2Cl_2 and stirred for 30 min. The solution was concentrated under reduced pressure, coevaporated with toluene and subsequently added to the acyl azide. After 16h stirring, the solution was diluted with CH_2Cl_2, washed with 1M HCl, $NaHCO_3$ (sat. aq.) and H_2O, dried over Na_2SO_4 and concentrated which gave fully protected peptide. LC/MS: R_t 11.21 min; linear gradient 10→90% B in 13.5 min; ESI/MS: *m/z* = 857.20 $(M+H)^+$. The crude peptide was dissolved in 50% TFA/CH_2Cl_2. After 30 min, TLC-analysis showed complete conversion of the starting material and the solution was concentrated, coevaporated. Silica gel purification (CH_2Cl_2 → 6% MeOH/CH_2Cl_2) afforded title compound **5** (63%, 35.0 mg, 47 μmol). LC/MS: R_t 7.89 min; linear gradient 10→90% B in 13.5 min; ESI/MS: *m/z* = 745.13 $(M+H)^+$. ^{1}H NMR (400 MHz, $CDCl_3$/MeOD) δ ppm 8.05 (d, *J* = 8.3 Hz, 1H), 7.96 (d, *J* = 8.5 Hz, 1H), 7.86 (d, *J* = 7.6 Hz, 1H), 7.78 (d, *J* =

8.4 Hz, 1H), 7.70 (d, *J* = 8.1 Hz, 1H), 7.00 (d, *J* = 8.5 Hz, 1H), 6.73 (d, *J* = 8.5 Hz, 1H), 4.74 (dt, *J* = 8.1, 8.1, 5.6 Hz, 1H), 4.61-4.55 (m, 1H), 4.43 (dd, *J* = 8.4, 4.6 Hz, 1H), 4.33 (dt, *J* = 8.4, 8.3, 3.9 Hz, 1H), 4.09 (dq, *J* = 6.3, 6.3, 6.3, 5.0 Hz, 1H), 3.30 (d, *J* = 5.0 Hz, 1H), 3.02 (dd, *J* = 14.0, 5.3 Hz, 1H), 2.92 (d, *J* = 5.0 Hz, 1H), 2.85 (dd, *J* = 14.1, 8.3 Hz, 1H), 1.92-1.83 (m, 1H), 1.83-1.75 (m, 1H), 1.74-1.65 (m, 1H), 1.60-1.46 (m, 6H), 1.36 (ddd, *J* = 13.8, 10.6, 4.2 Hz, 1H), 1.17 (d, *J* = 6.4 Hz, 3H), 1.15-1.04 (m, 2H), 0.98-0.83 (m, 18H). ^{13}C NMR (100 MHz, $CDCl_3$/MeOD) δ ppm 209.0, 172.7, 172.6, 172.2, 171.1, 168.5, 156.3, 130.6, 127.4, 115.8, 67.6, 59.6, 58.7, 58.6, 58.5, 55.0, 52.8, 52.4, 51.2, 39.7, 37.7, 37.5, 37.2, 25.6, 25.4, 25.3, 23.5, 21.3, 18.9, 16.9, 15.5, 15.5, 11.3, 11.2. FT-IR: ν_{max}(neat)/cm^{-1} 3285.8, 2965.4, 2108.9, 1635.8, 1517.0, 1452.2, 1383.3, 1220.3, 832.2. HRMS: ($M+H^+$) calcd for $C_{36}H_{56}N_8O_9$ 745.42430 found 745.42470.

Biological evaluation

Labeling of HEK293T lysate in the presence of hexosaminidase inhibitors NAG-thiazoline

To prevent removal of the O-GlcNAc residue of probes **1-4** by hexosaminidases present in the cell lysate, HEK293T lysate was treated with probes **1-4** in the presences broad spectrum hexosaminidase NAG-thiazoline. Comparison with untreated samples showed no evident differences (Figure 1).

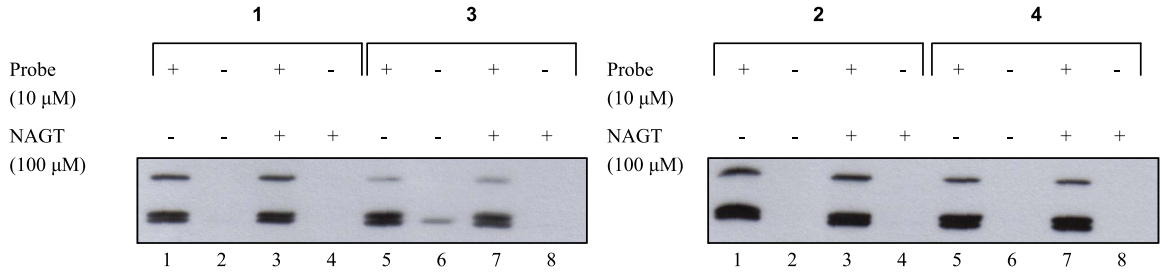


Figure 1. Streptavidin blot of HEK293T lysate (10 μg) treated with probes **1-4** in the presences of NAG-thiazoline (Lanes 3, 4 and 7, 8), and in the absences of NAG-thiazoline (Lanes1, 2 and 5, 6).

Competition experiment with MV151

To 9μL of human embryonic kidney 293 T (HEK) cell lysate (1 μg/μL in 50 mM Tris buffer pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP) was added 1 μL of a concentration series of probes **1-4** and the mixture was incubated for 1h at 37°C. Next, 1 μL of MV151 (1 μM final concentration) was added and the solution was incubated for 1h at 37°C. The reaction was quenched by the addition of 4× Laemmli sample buffer (6 μL) containing β-mercaptoethanol followed by boiling for 3 min. The proteins were resolved on 12.5% SDS-PAGE. Fluorescence was measured in the wet gel slabs on a Typhoon Variable Mode Imager (Amersham Biosciences) using the CY3/Tamra settings (λ_{ex} 532 λ_{em}560).

Labeling of the proteasome with probes 1-4

HEK-cell lysate (18μL, 1 μg/μL) was incubated with increasing concentrations of probes **1-4** (2 μL) for 1h at 37°C, after which biotine modified phosphine (2 μL, 400 μM final concentration) was added. After 1h at 37°C, the proteins were denatured by the addition

4× Laemmli sample buffer (6 μL) followed by boiling for 3 min. The proteins were resolved on 12.5% SDS-PAGE and transferred to a PVDF-membrane. The membranes were blocked with 0.5% bovine serum albumine in TBS-TWEEN (0.1% TWEEN-20) for 30 min and incubated with Streptavidine-HRP (Amersham Bioscience 1:5000) for 30 min at ambient temperature. The membranes were briefly washed with TBS containing 0.1% TWEEN-20 and TBS followed by visualisation of the biotinylated proteins with an ECL+ Kit (Amersham Bioscience).

Labeling of the proteasome with 1-4 in living cells

Human embryonic kidney cells (1×10^6) were cultured in 6-well plates in DMEM containing 10% fetal calf serum, 10 units/mL penicillin and 10 μg/mL streptomycin in a 7% CO_2 humidified incubator at 37°C overnight. The medium was withdrawn, the indicated amount probe **1-4** in DMSO (10 μL, 100× stock) was added to the medium (1 mL), after which the medium was added to cells. The cells were incubated for 16h with the probes. Thereafter, the medium was removed; the cells were washed with PBS and harvested. After flash freezing in N_2 (liquid), the cells were resuspended in 4 volumes of homogenation buffer (50 mM TRIS pH 7.5, 250 mM Sucrose, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 0.025% digitonin), sonicated (12W, 20 sec.) and centrifuged at 16.000 rcf at 4°C for 20 min. The supernatant was collected and the protein concentration was determined using Bradford analysis. In case of a competition experiment, some 10 μg of protein was exposed to MV151 (1 μM) for 1h at 37°C and subsequently treated as described previously for competition experiments in lysates. In case of a labeling

experiment, whole lysates (10 μg) were incubated with staudinger-bertozzi biotin phosphine reagent (400 μM) for 1h at 37°C and visualization as described before.

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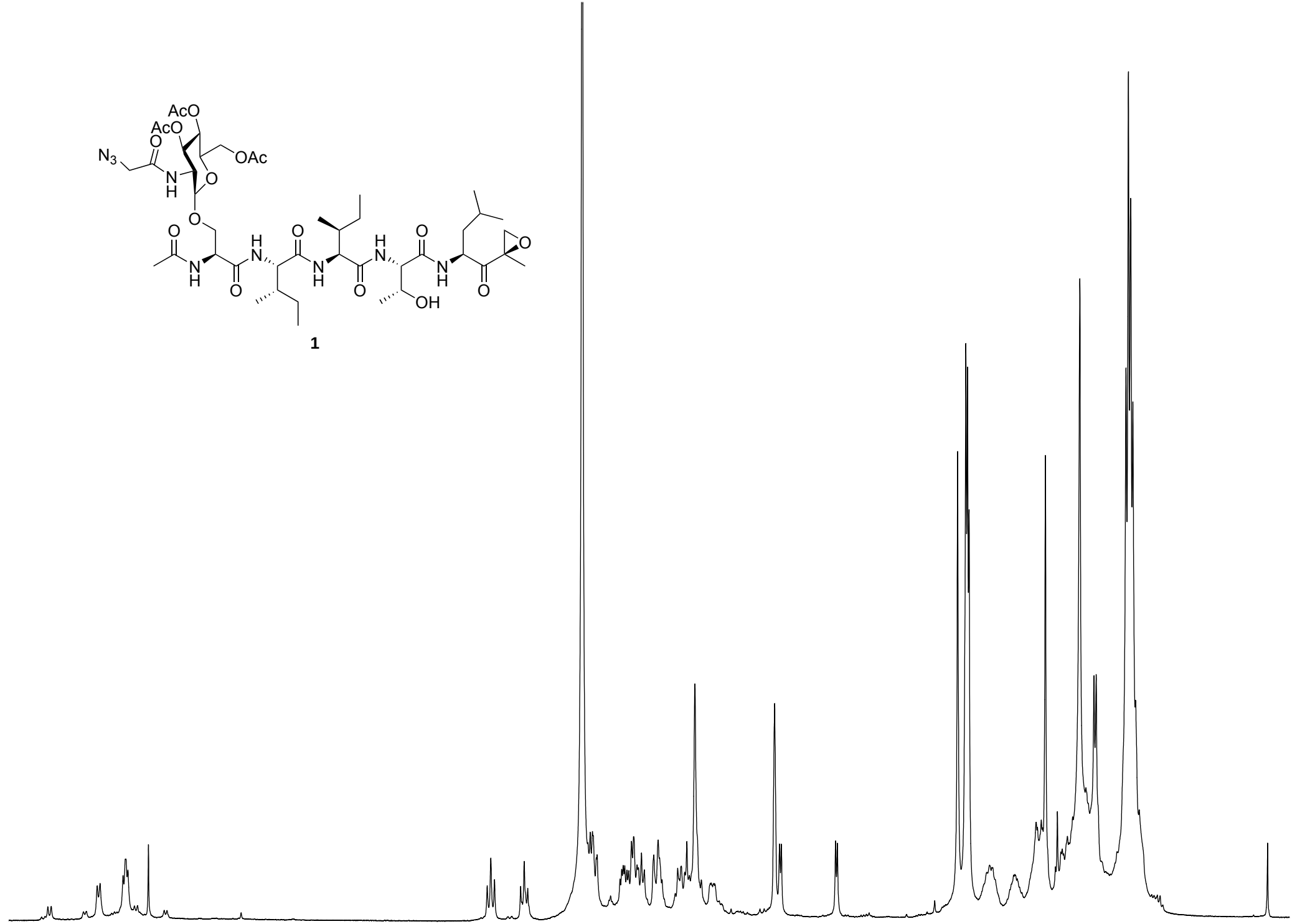
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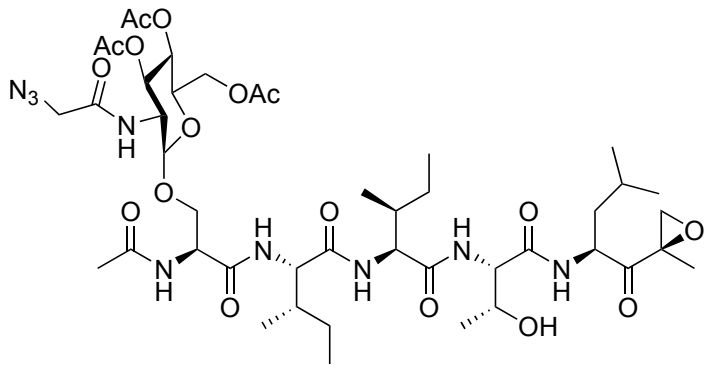
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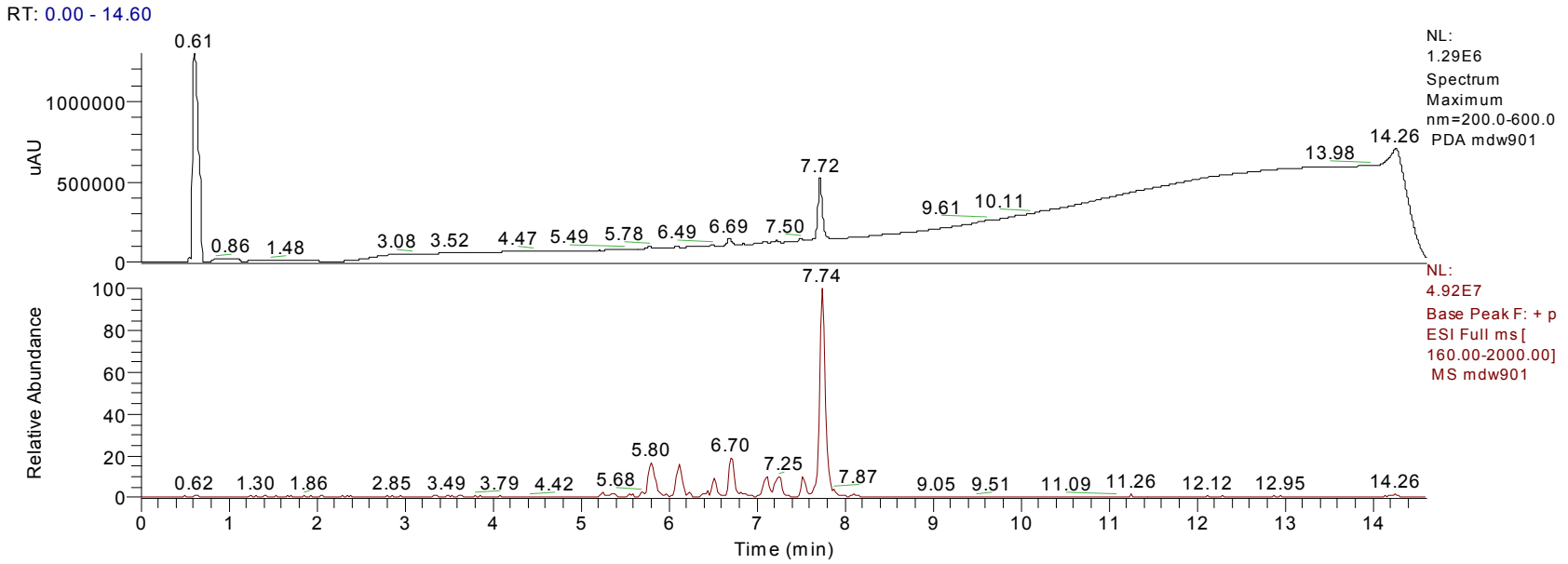


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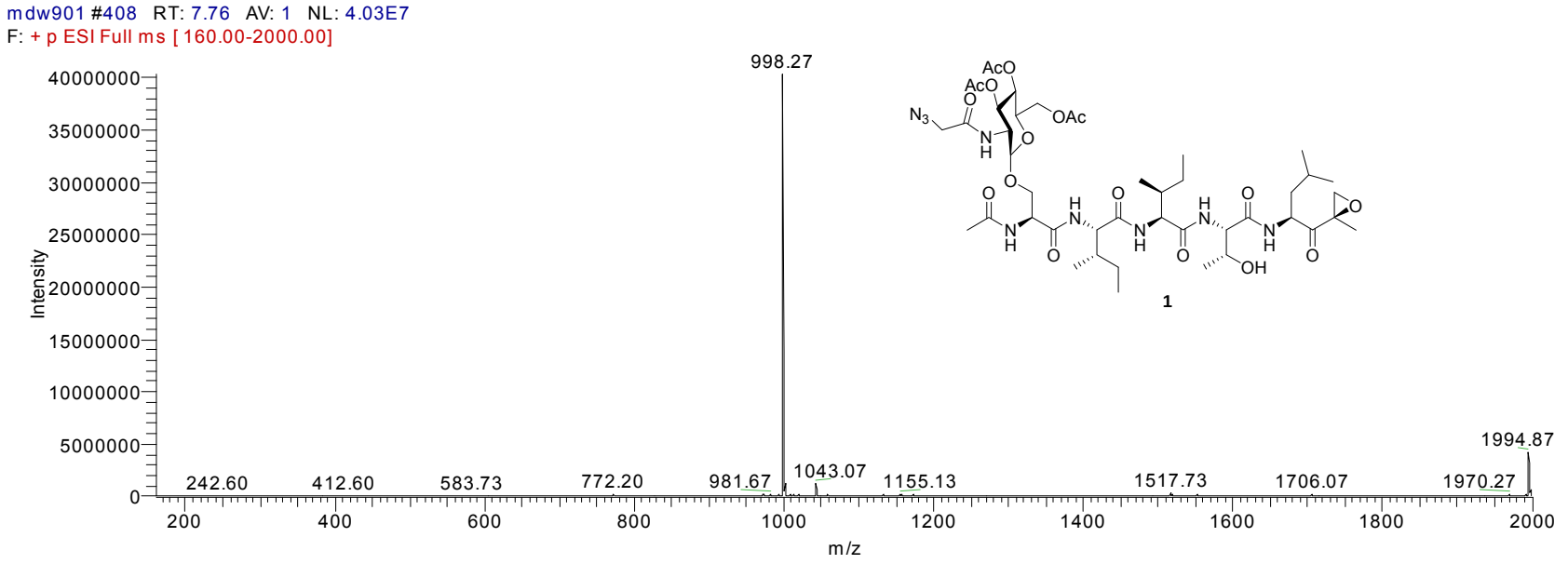

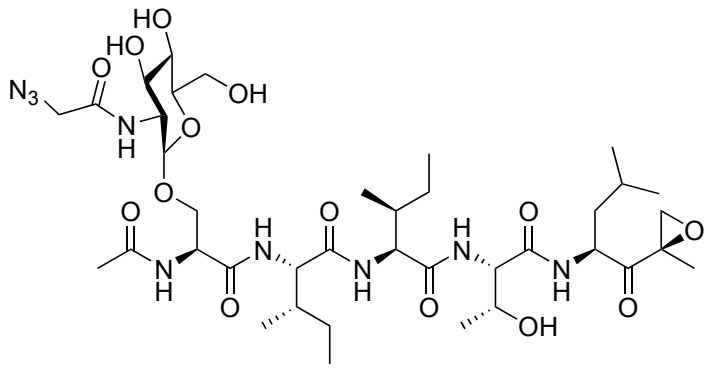

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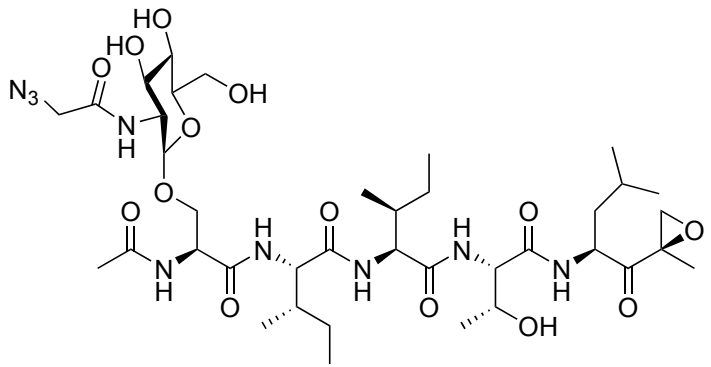

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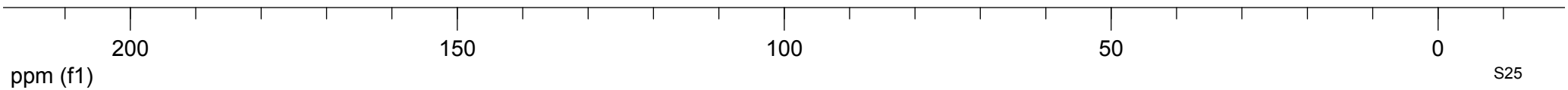

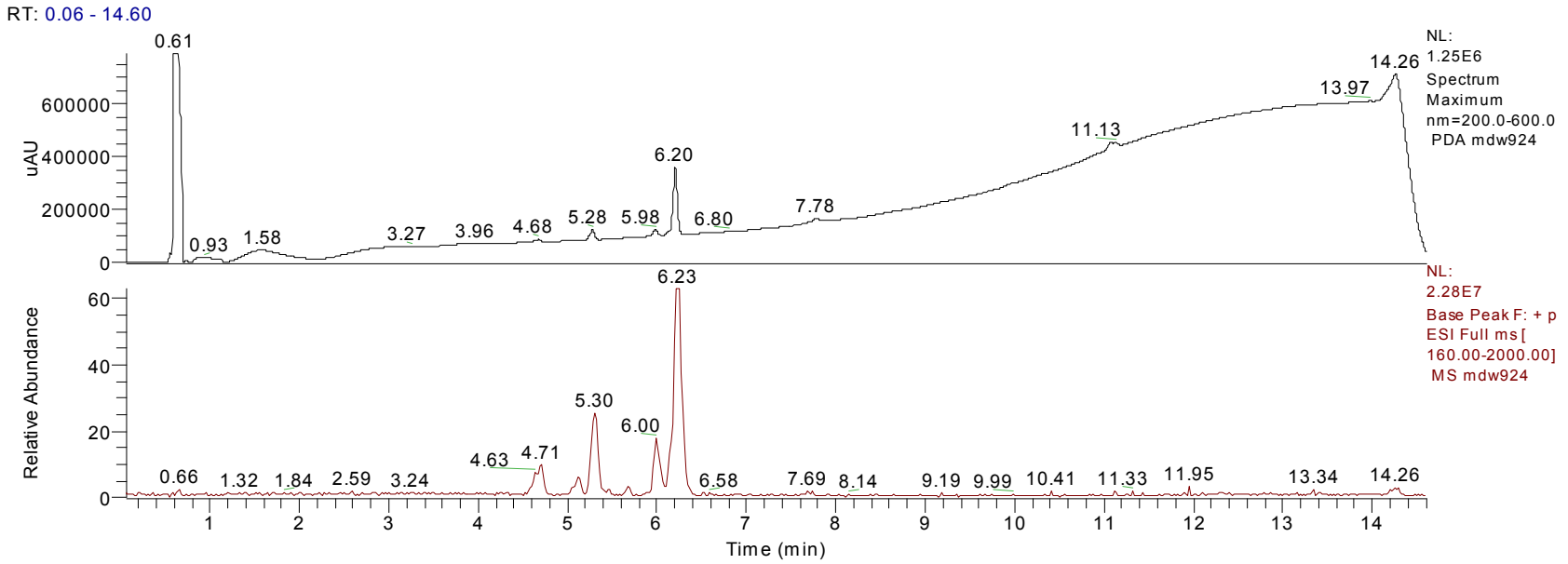


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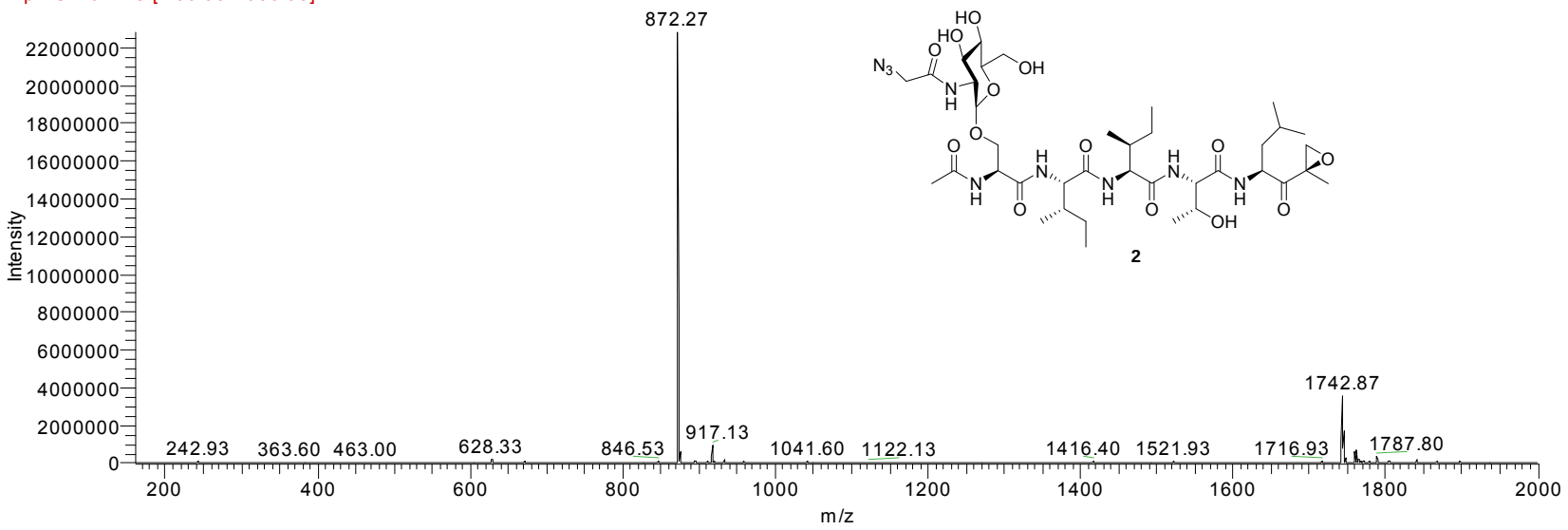

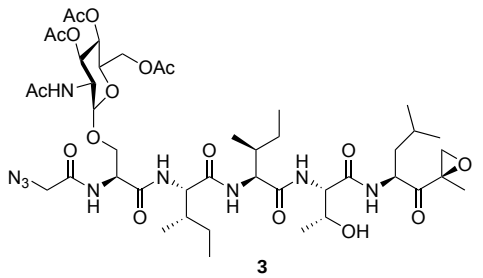

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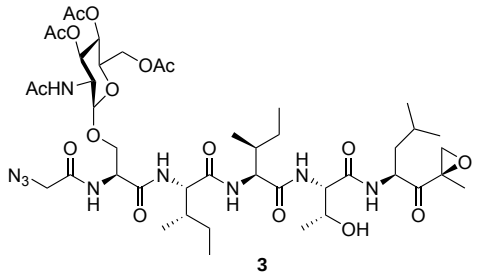
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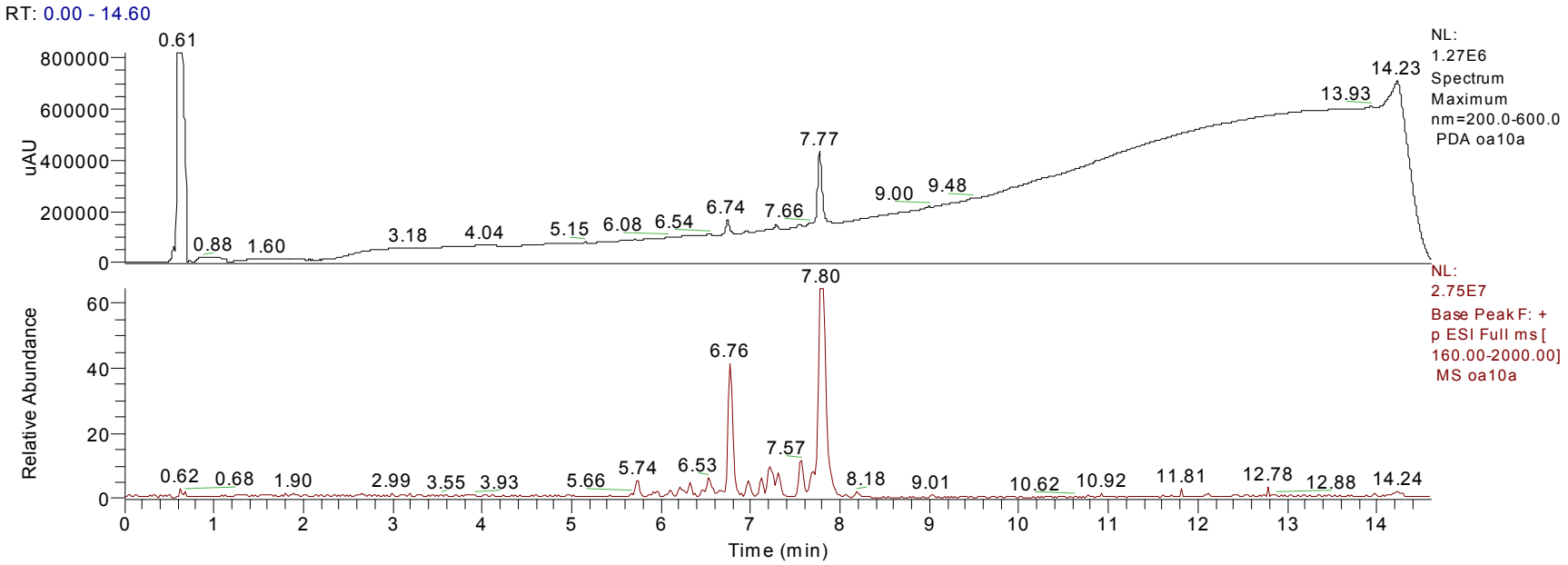


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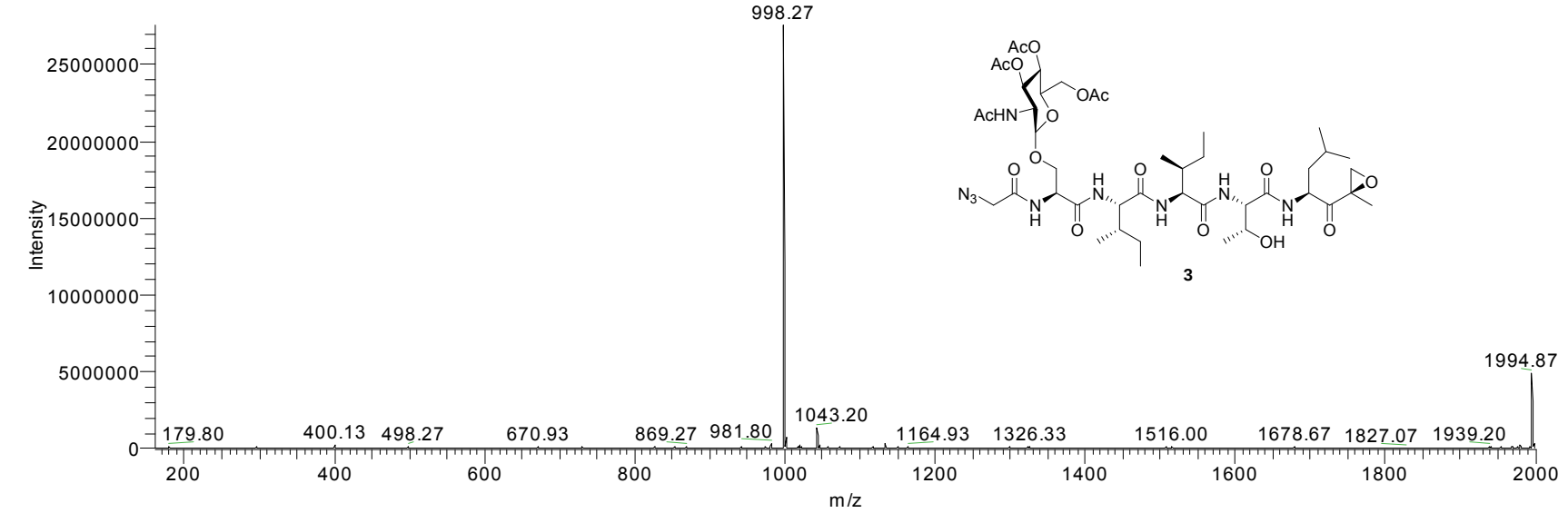

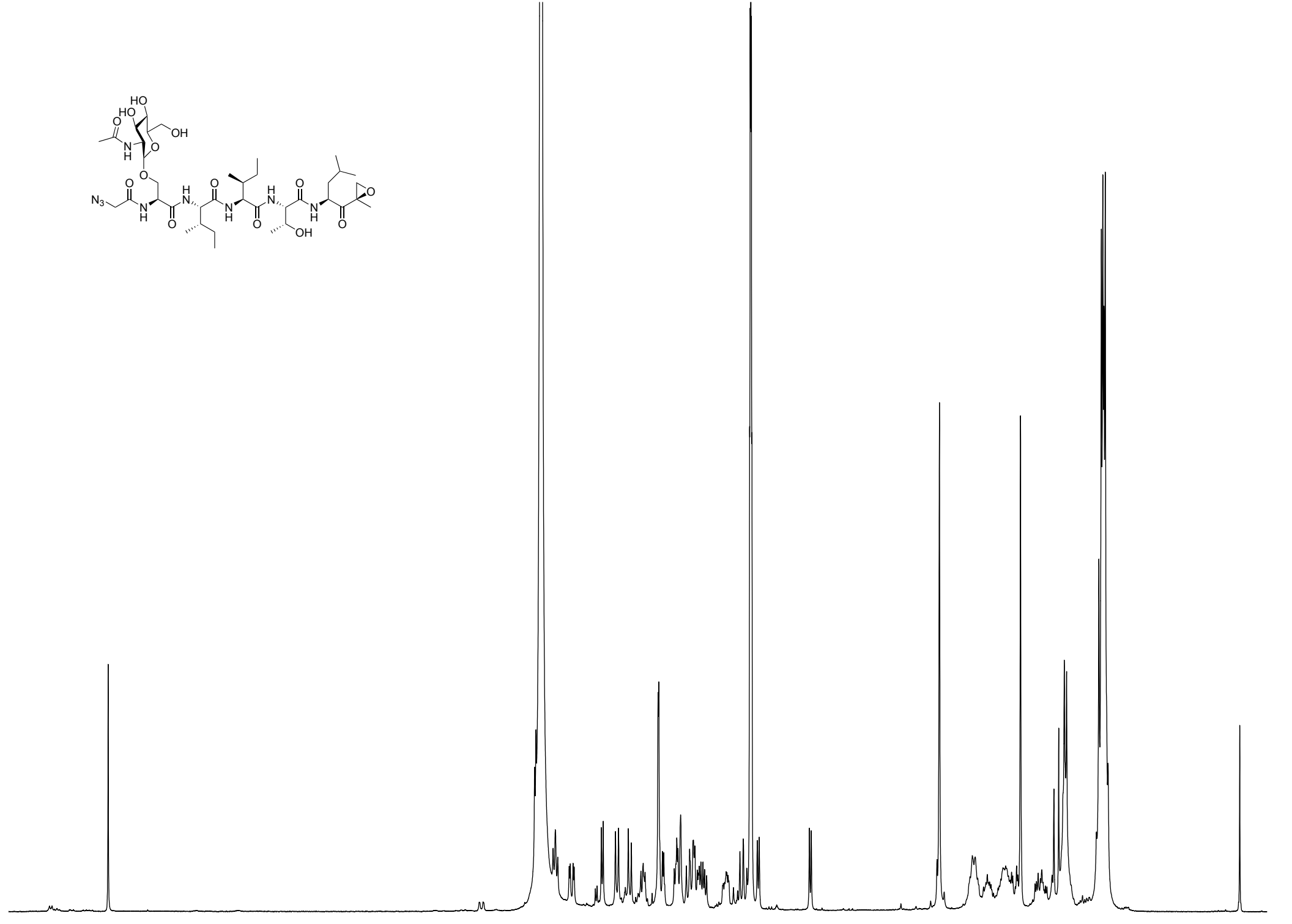
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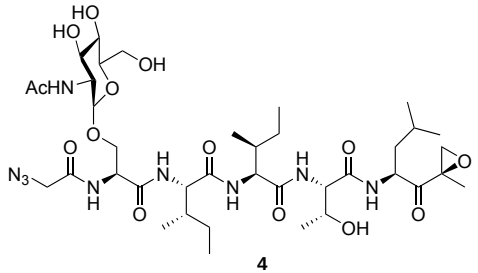
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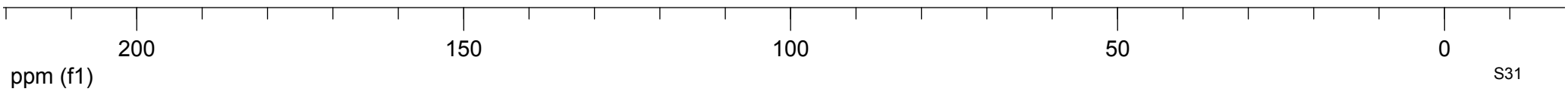
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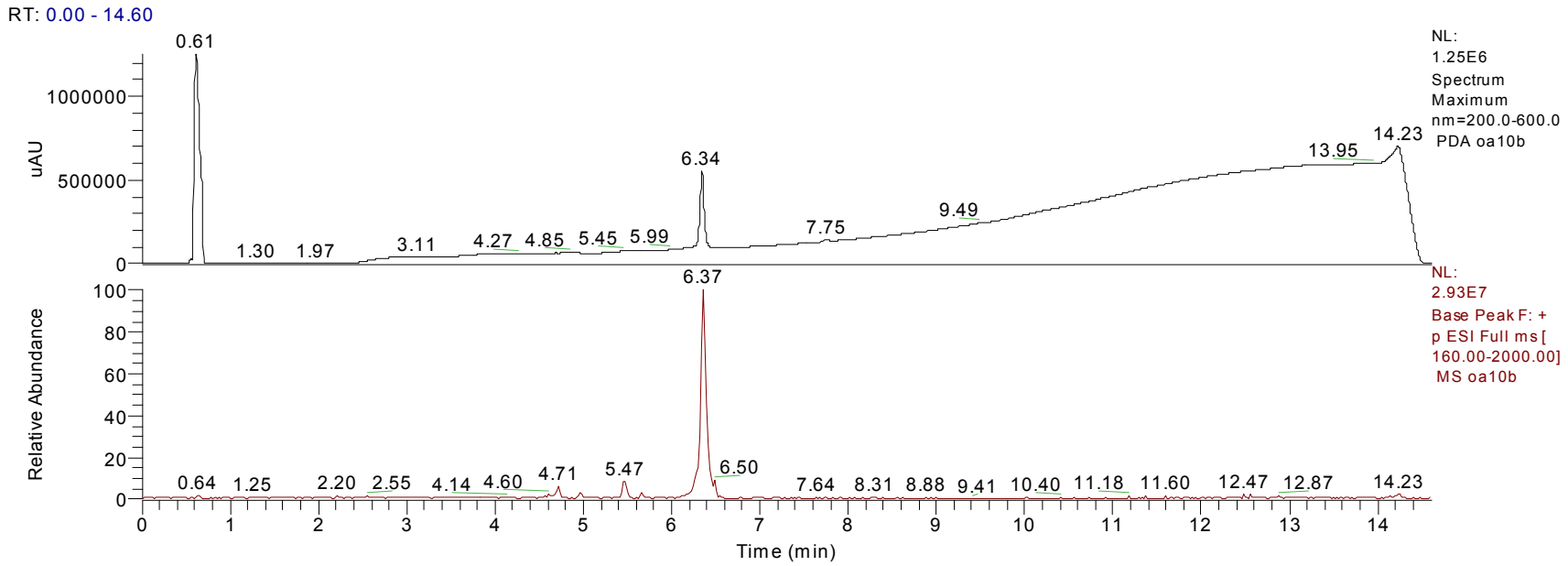


```
Instrument Method:     C:\Xcalibur\methods\General(TFA)\1090%B00_C=TFA_20ul_+pf 15min.meth
Processing Method:
Vial:                  a:19
Injection Volume (µl):    5.00
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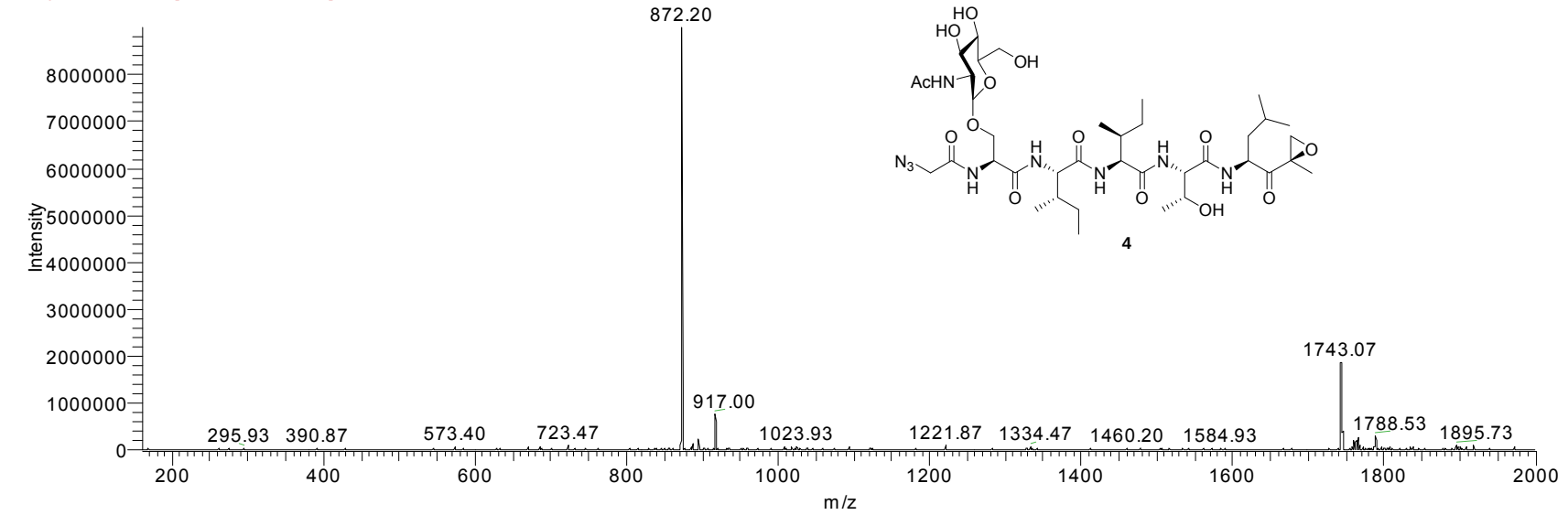
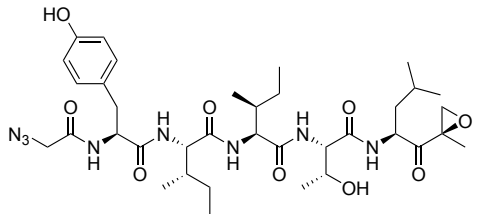
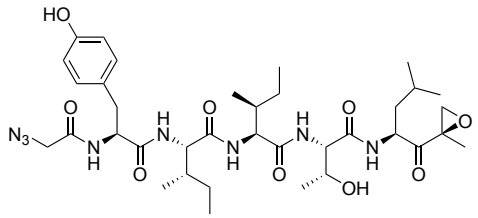




5



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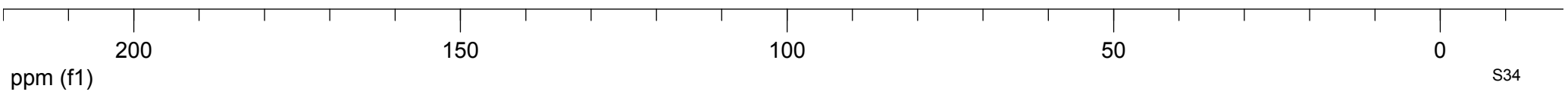
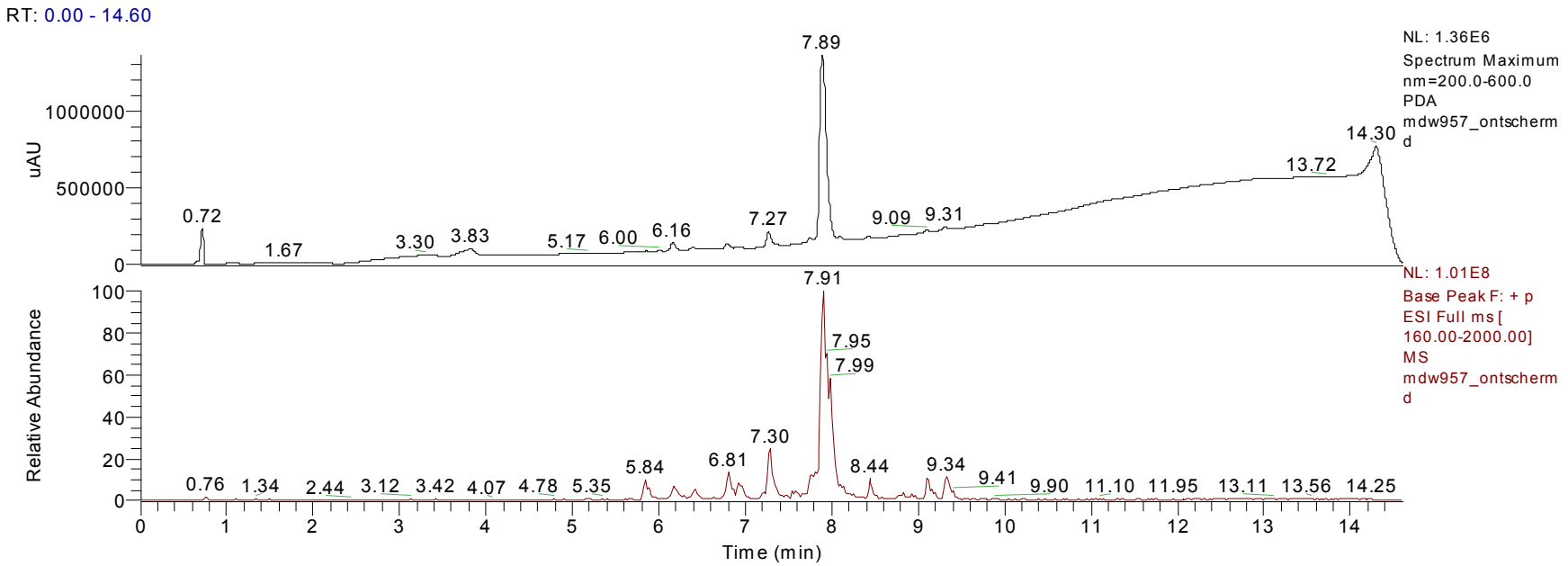
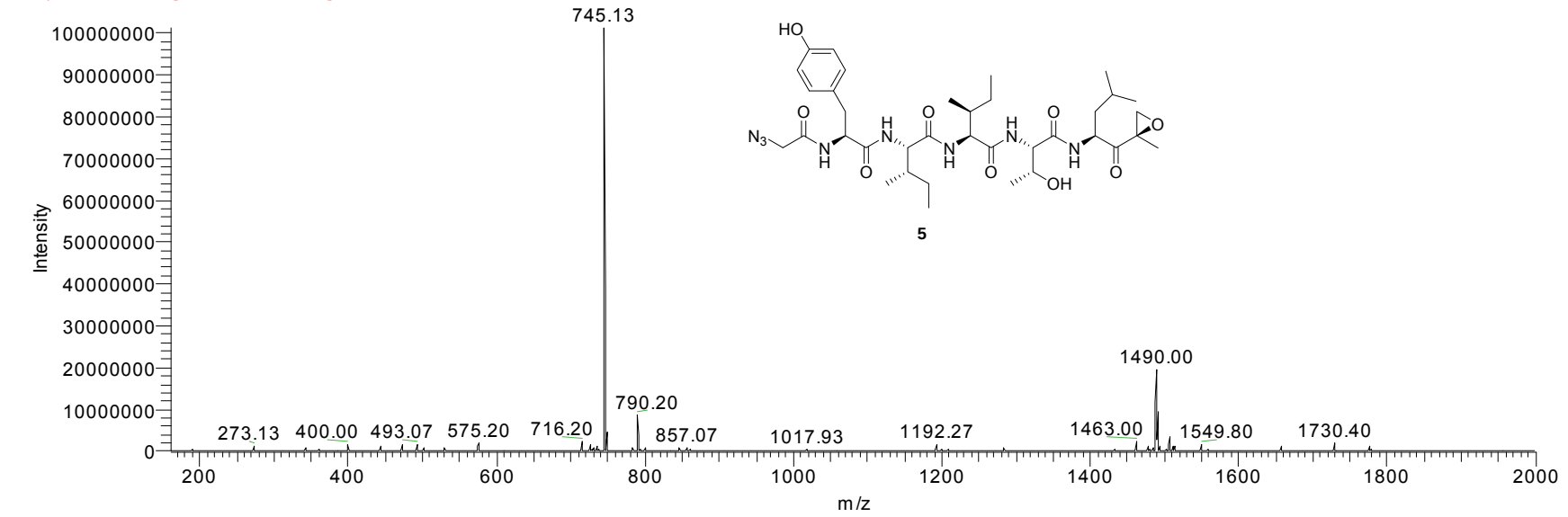
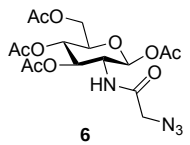





```
Instrument Method:     C:\Xcalibur\methods\General(TFA)\1090%B10_C=TFA_20ul_+pf 15min.meth
Processing Method:
Vial:                  A:9
Injection Volume (µl):   10.00
```





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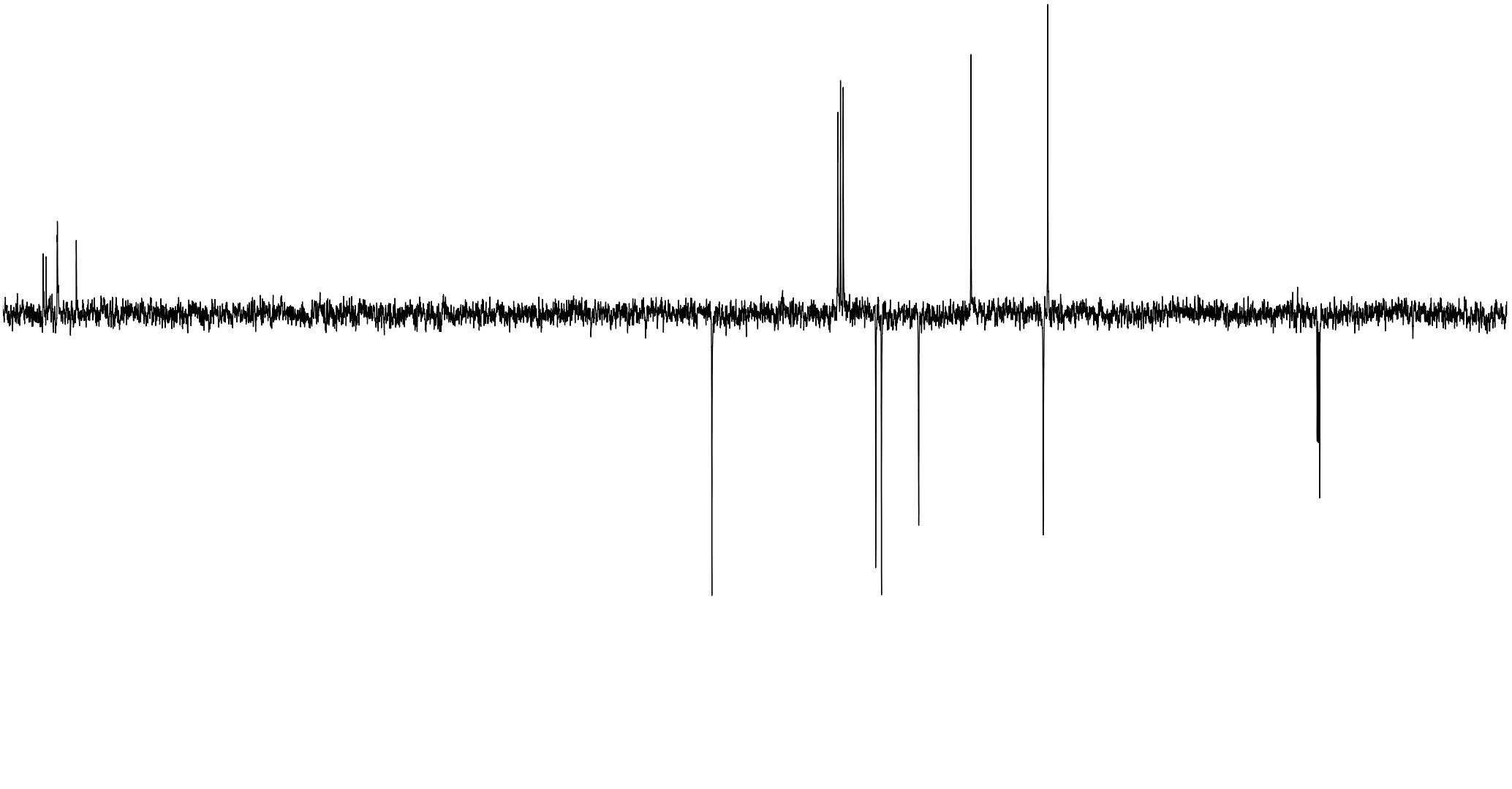
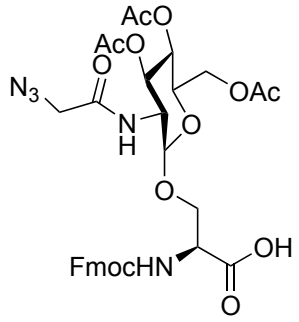
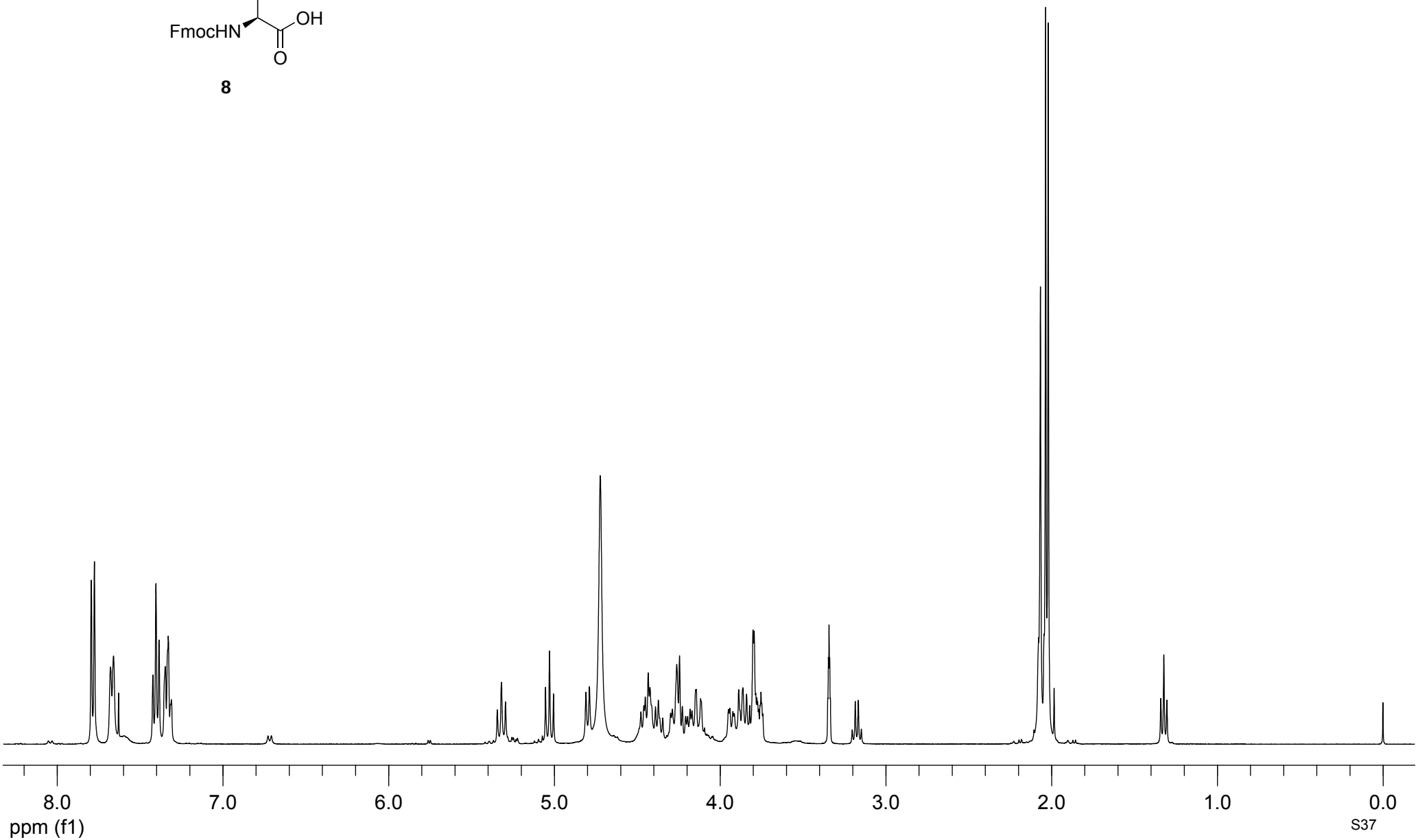
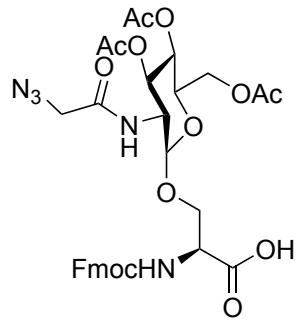




8





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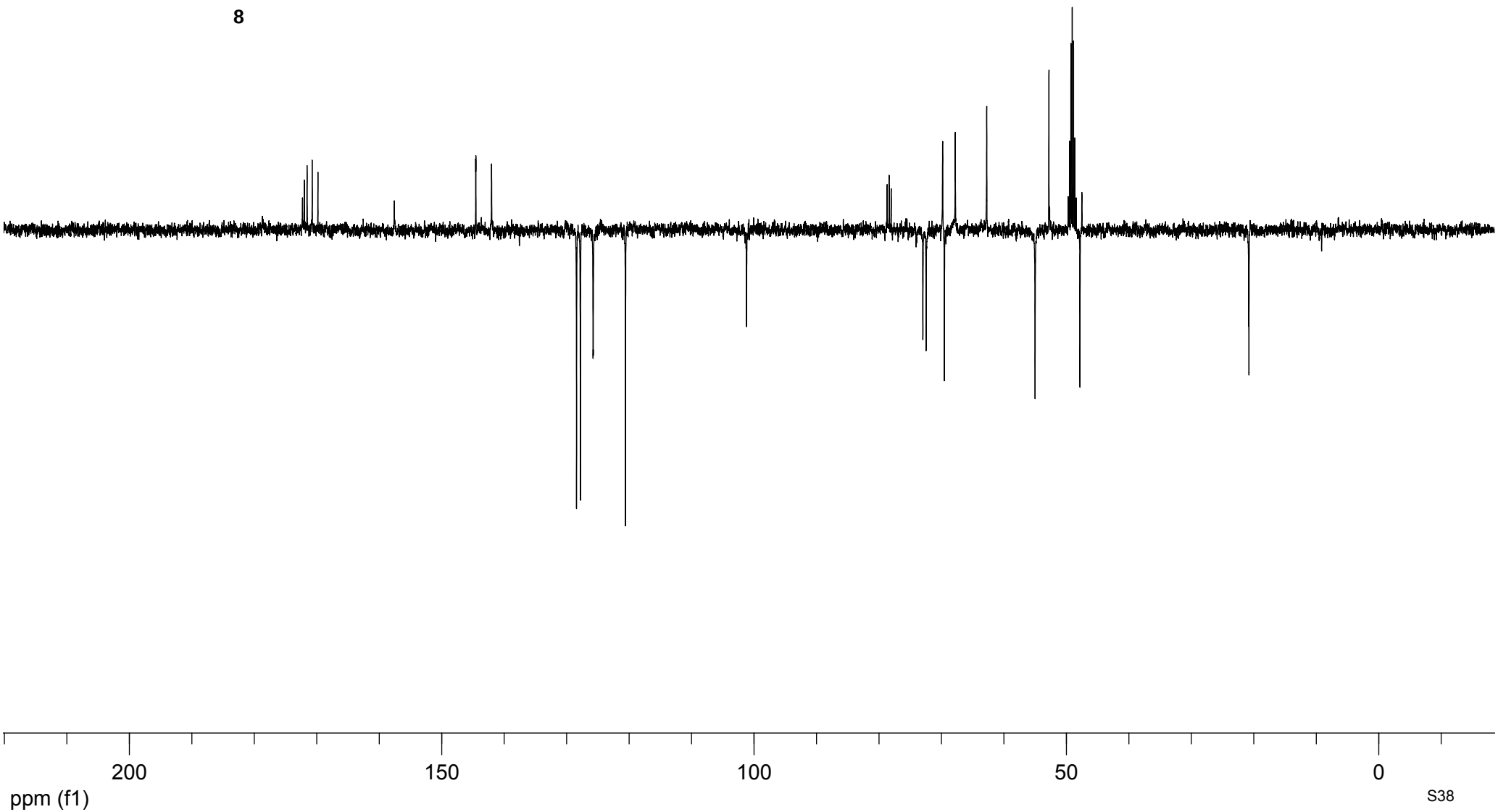
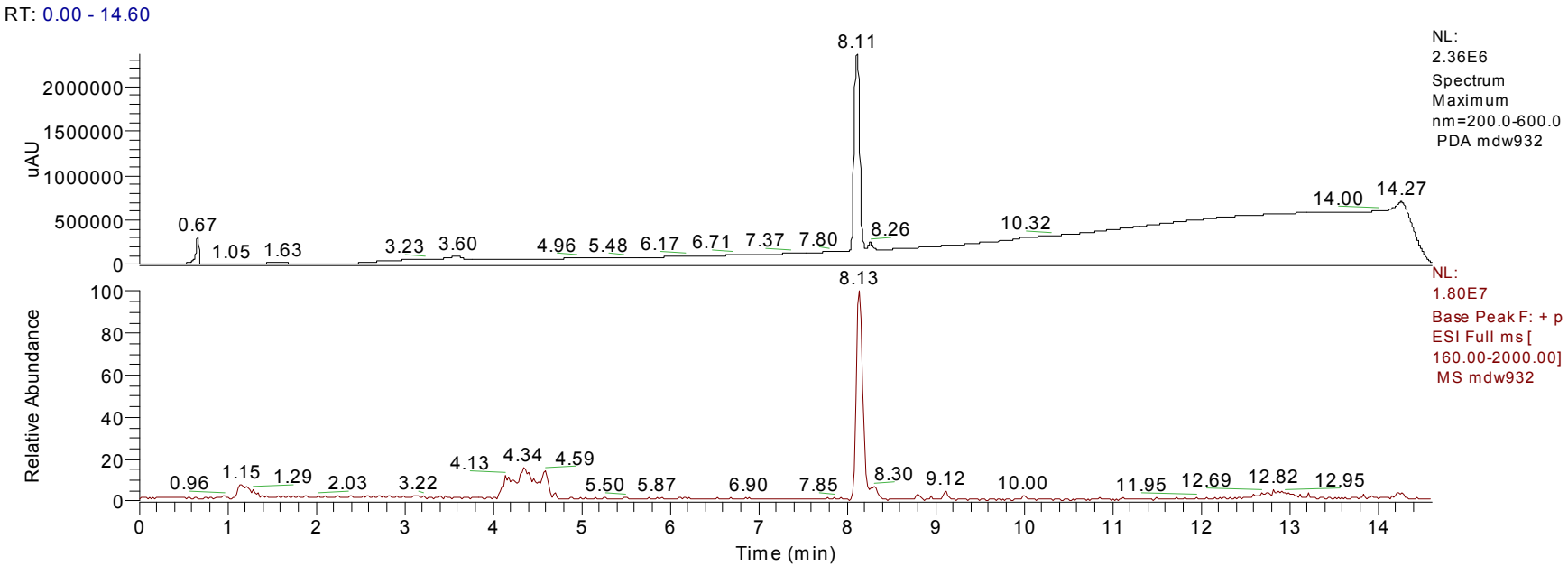
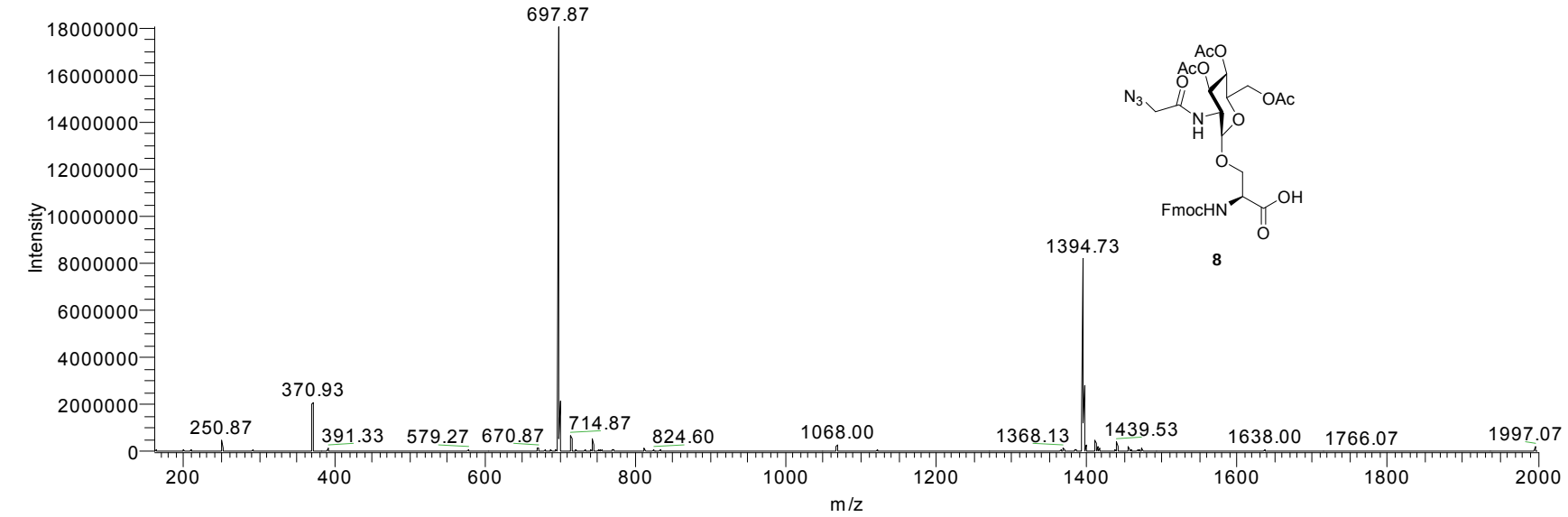
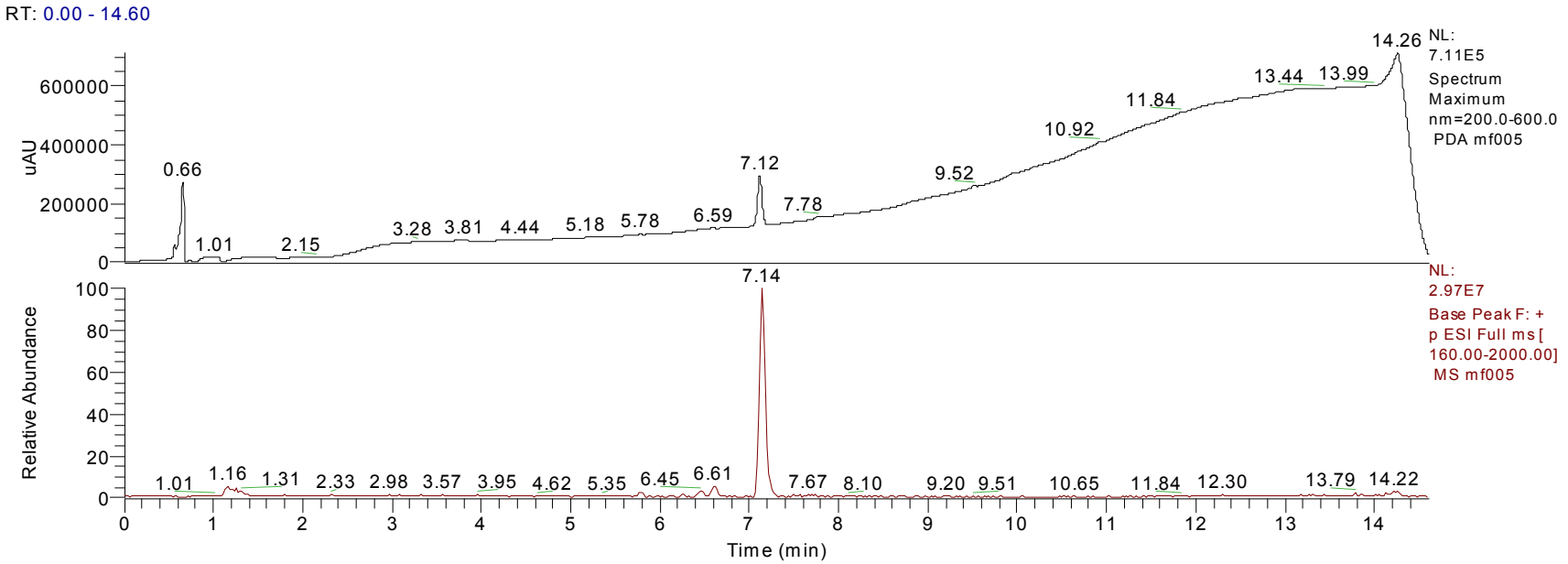


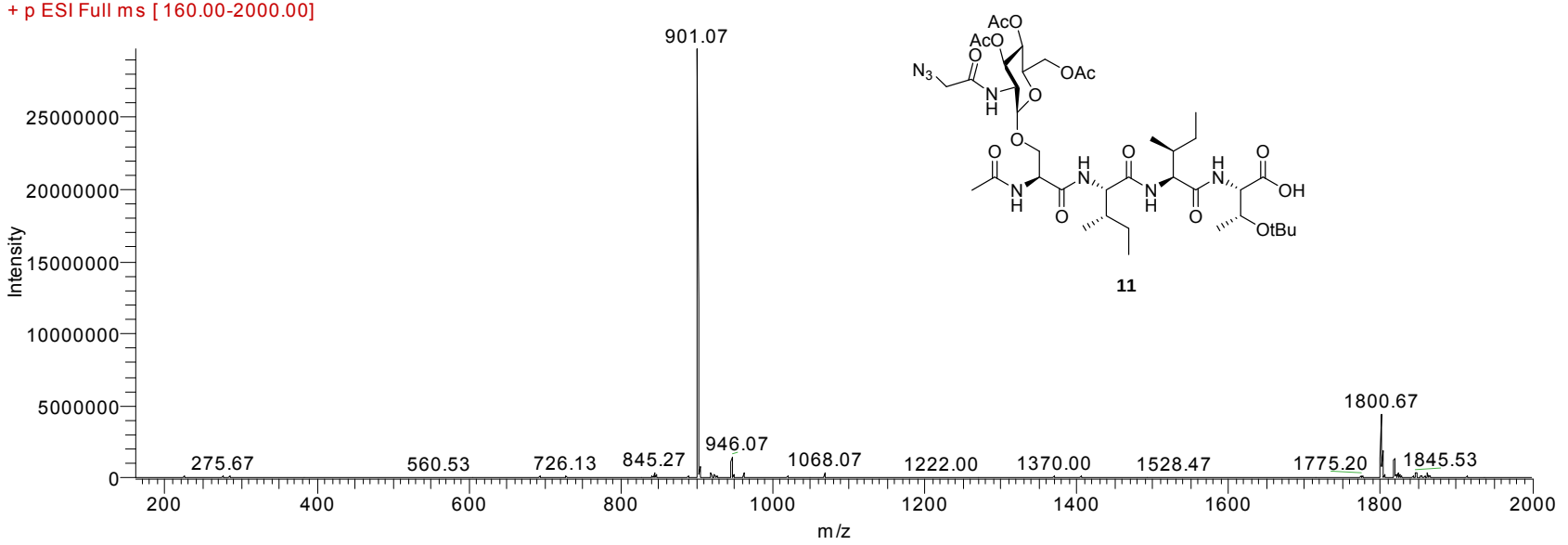



```
Instrument Method:     C:\Xcalibur\methods\General(TFA)\1090%B10_C=TFA_20ul_+pf 15min.meth
Processing Method:
Vial:                  A:27
Injection Volume (µl):   10.00
```

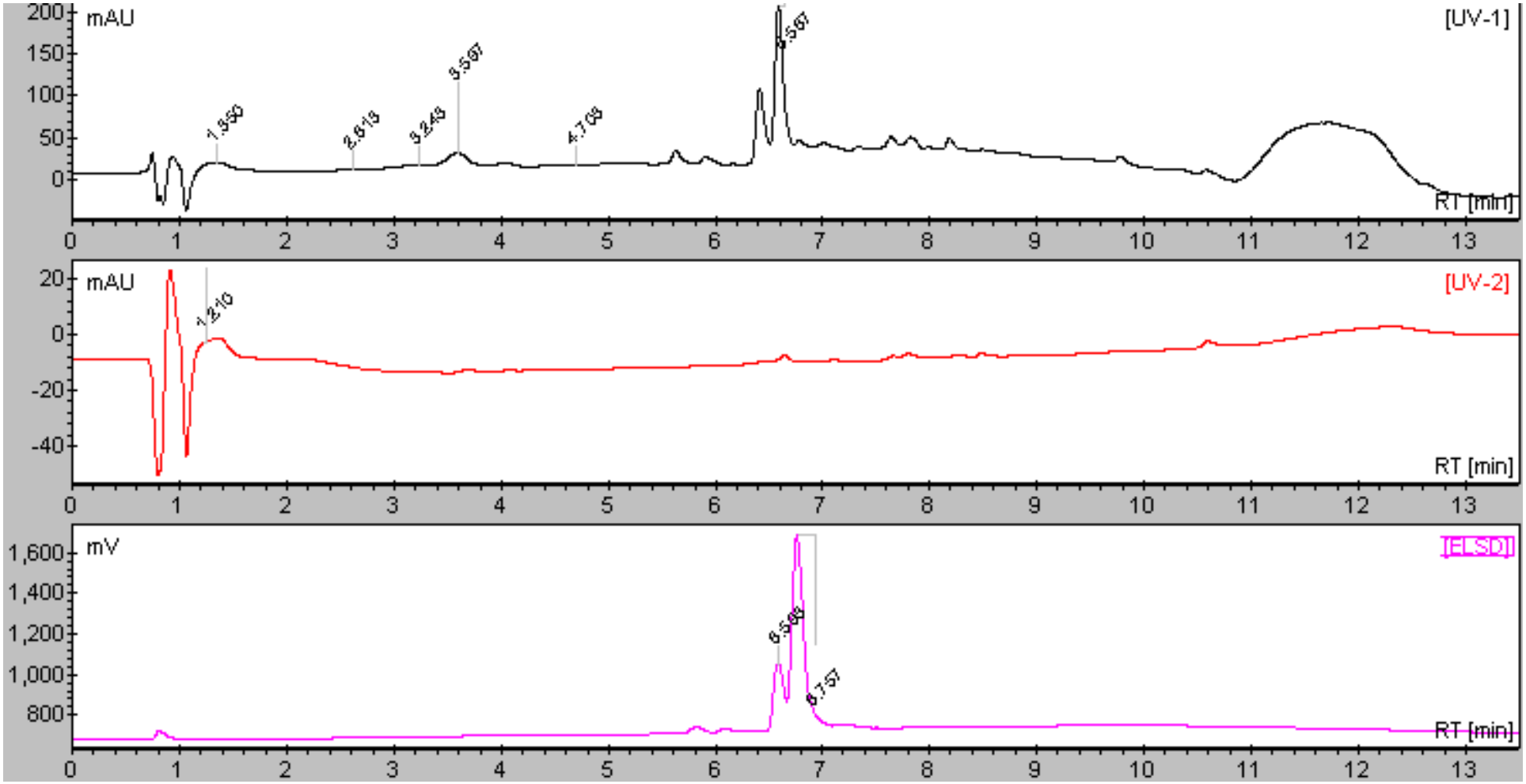


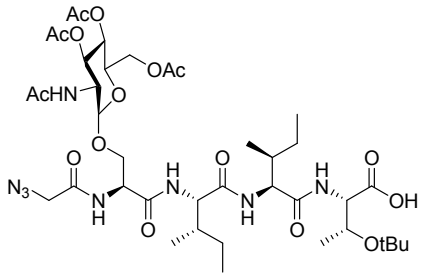



```
Instrument Method:     C:\Xcalibur\methods\General(TFA)\1090%B10_C=TFA_20ul_+pf 15min.meth
Processing Method:
Vial:                  A:29
Injection Volume (µl):   10.00
```

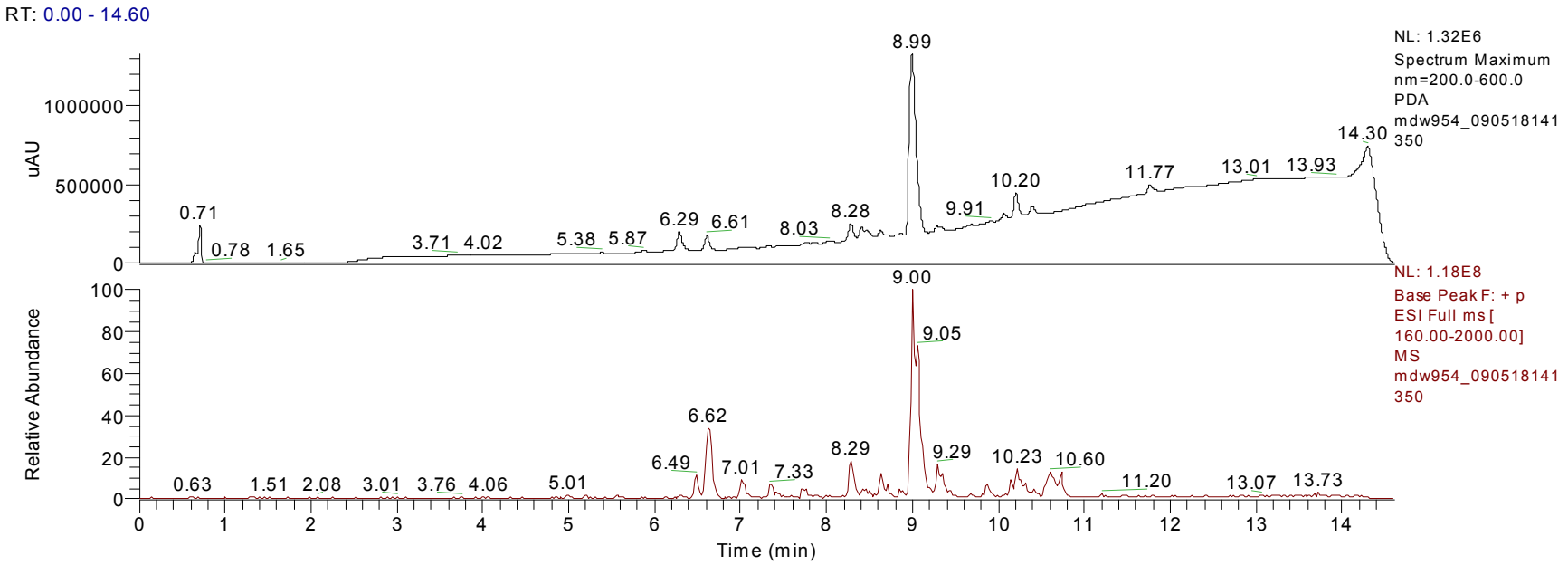


11


mAU
[UV-1]
1.350
2.613
3.248
3.567
4.709
6.567
RT [min]
mAU
[UV-2]
1.210
RT [min]
mV
[ELSD]
6.568
6.757
RT [min]


AcO
AcO
OAc
AcHN
O
O
N3
H
N
O
H
N
O
H
N
O
OH
OtBu

12




```
Instrument Method:     C:\Xcalibur\methods\General(TFA)\1090%B10_C=TFA_20ul_+pf 15min.meth
Processing Method:
Vial:                  A:20
Injection Volume (µl):   10.00
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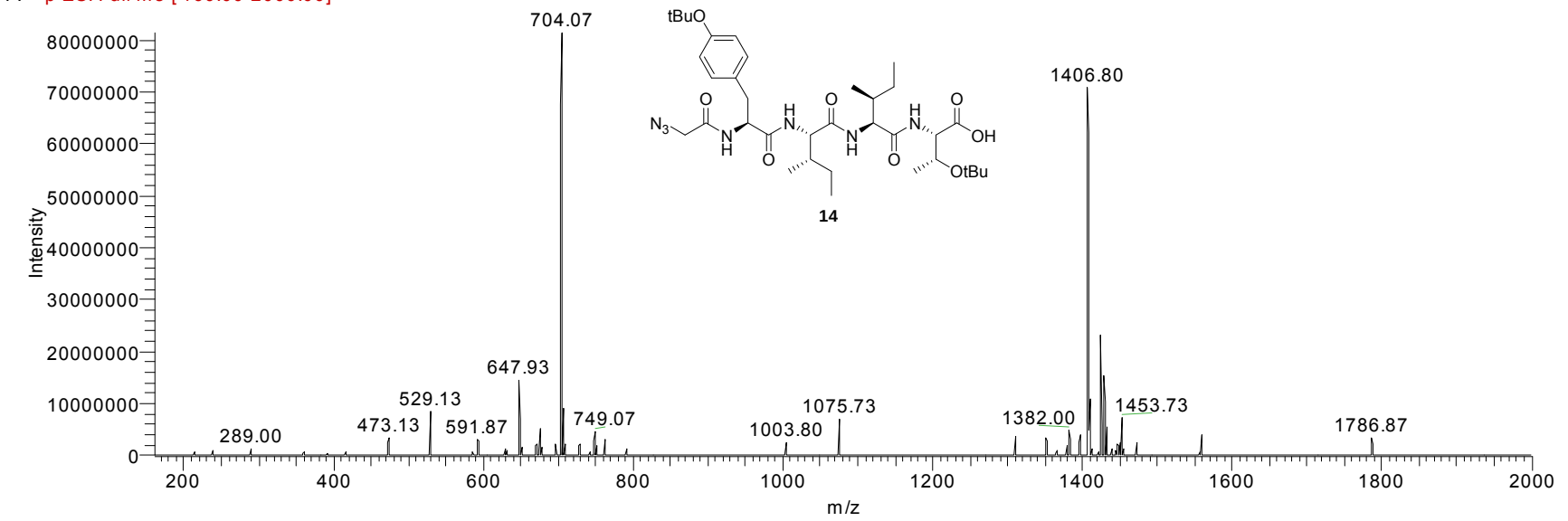
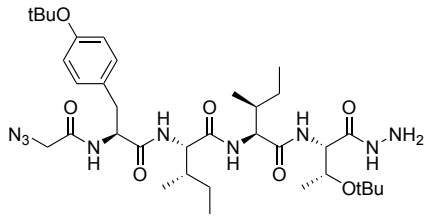
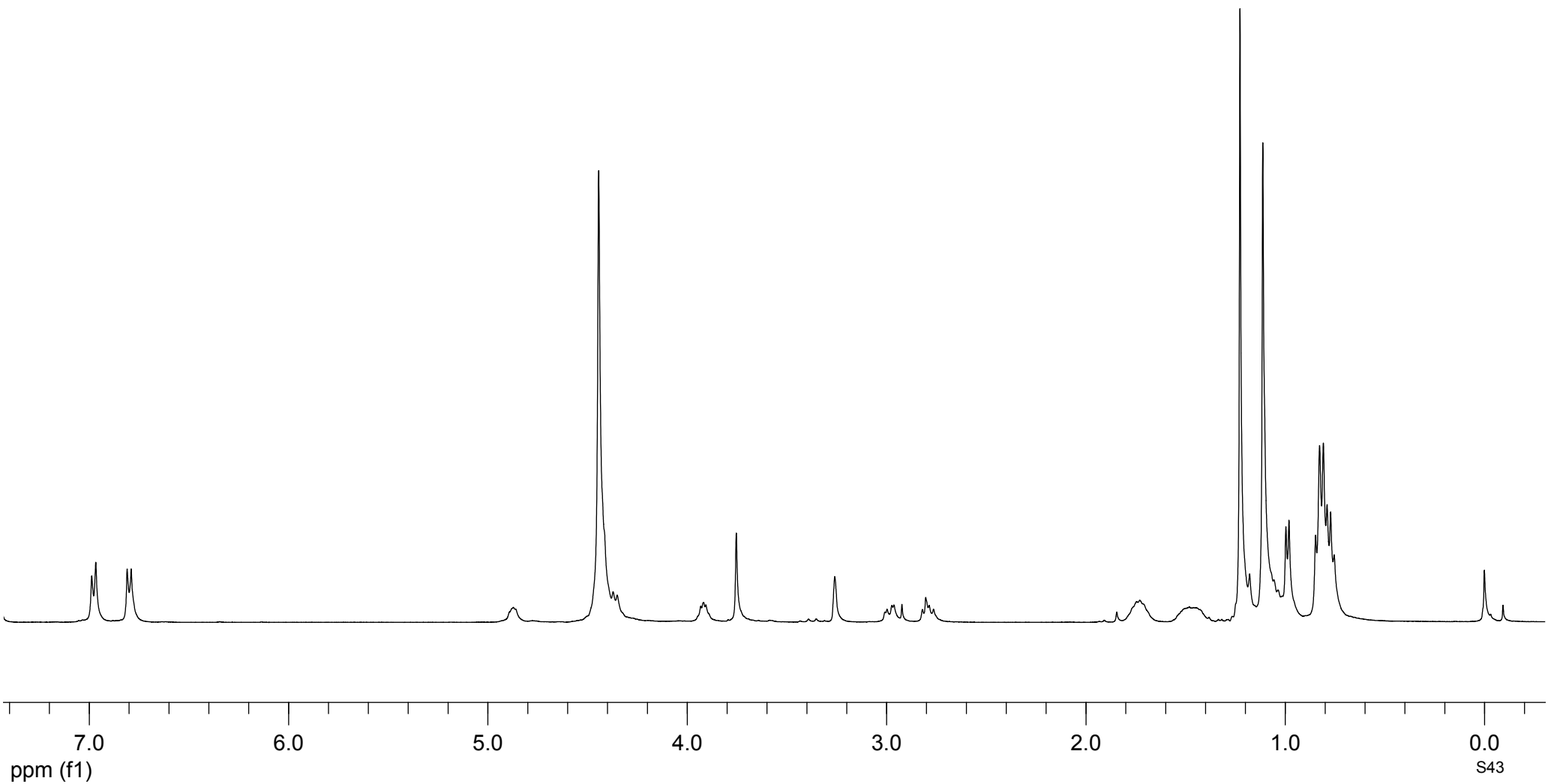
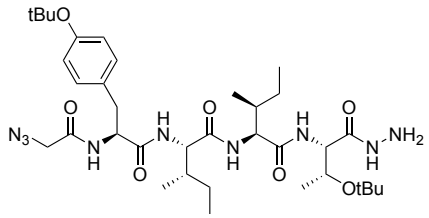




15





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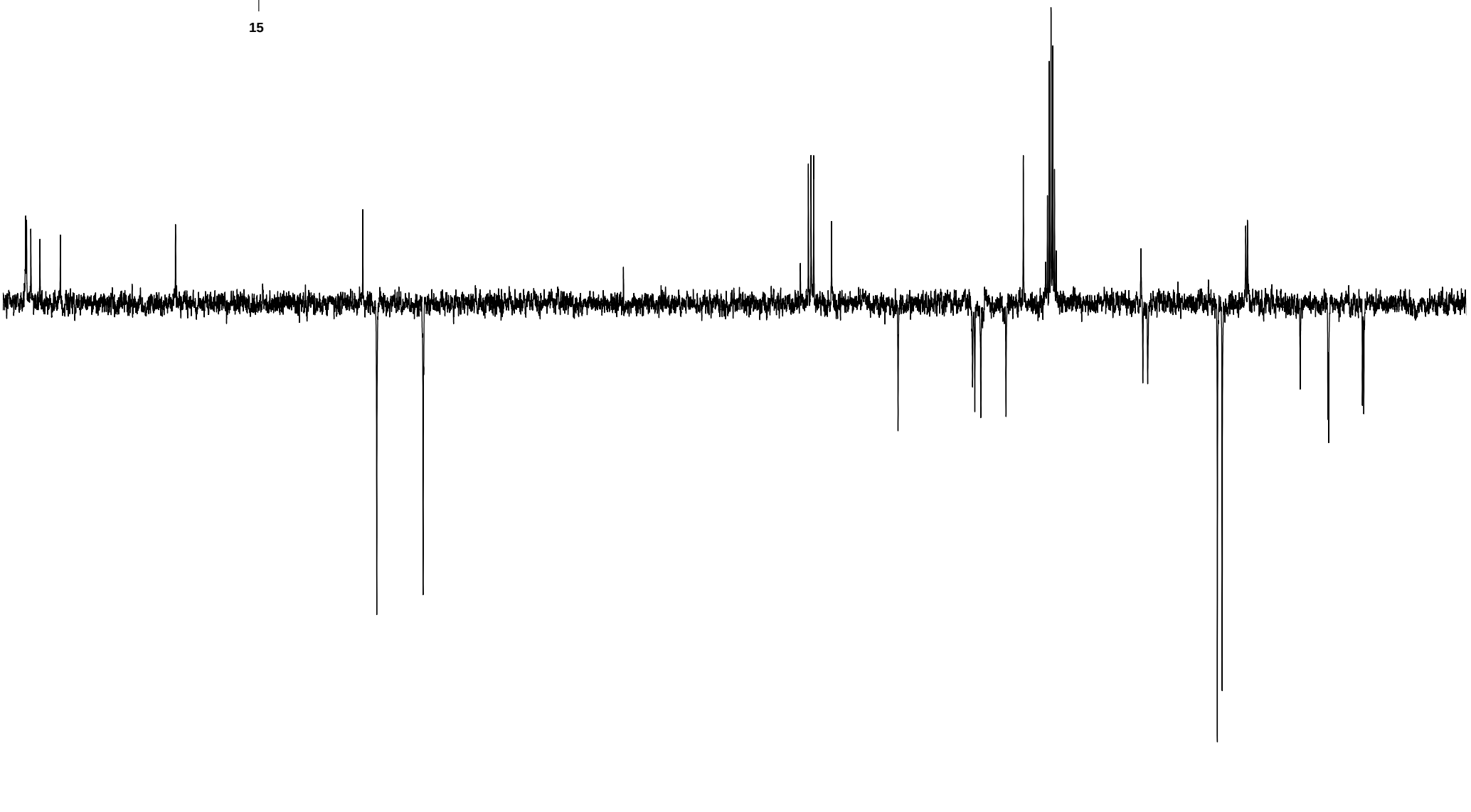

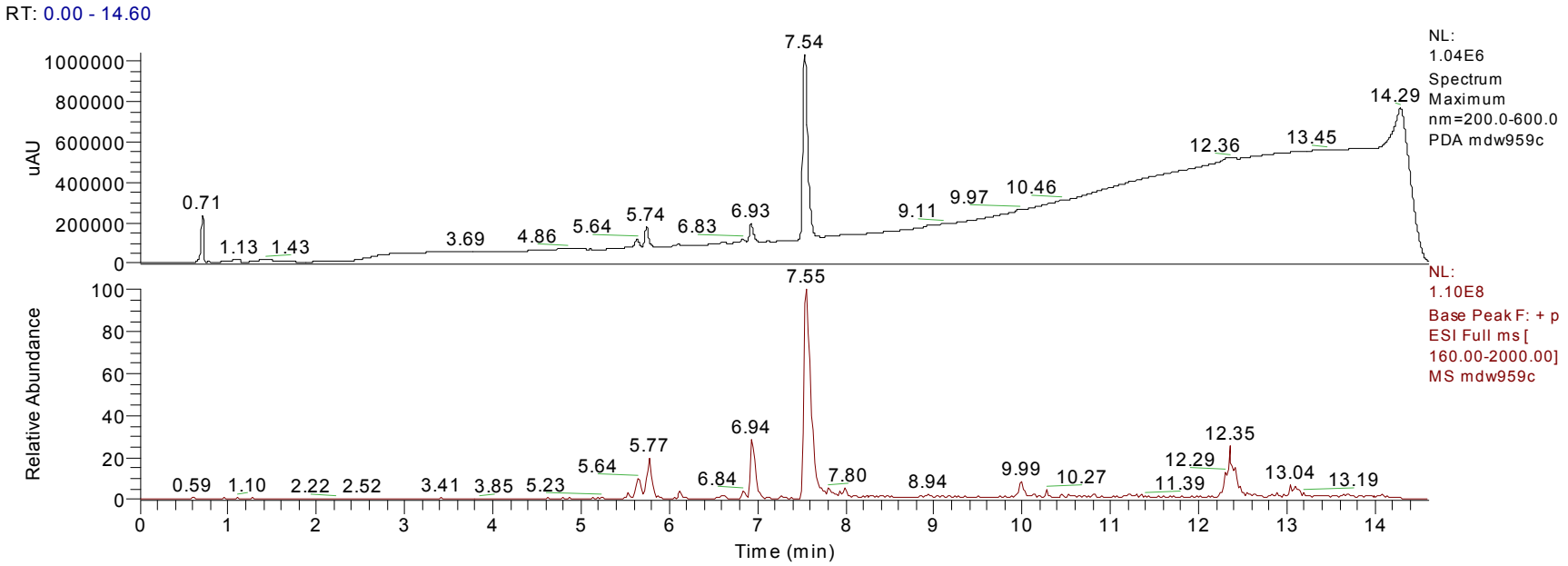


```
Instrument Method:     C:\Xcalibur\methods\General(TFA)\1090%B10_C=TFA_20ul_+pf 15min.meth
Processing Method:
Vial:                  A:23
Injection Volume (µl):   10.00
```